Development of a biosensor using aptamer/antigen interactions

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Abstract:

The use of aptamers acting within novel biosensors as molecular recognition elements is well documented with a wide variety of techniques being adapted to take advantage of the benefits of oligonucleotide detection. A continuing barrier to the commercial use of ssDNA or RNA aptamers remains the lack of a high-throughput system that confers reliable selection and description of suitable species. Here we describe simple methodologies that utilise fusion proteins, modified affinity chromatography, HPLC, and, nanopore assays, along with other techniques to isolate novel aptamers to well-characterised proteins. These methods have yielded novel aptamers to the HsdR, and HsdS subunits of type I restriction-modification system EcoR124I and to the human rhinovirus 3C protease, along with an enriched libraries for nitrotyrosine. In addition to the isolation of novel aptamer species, methods for the characterisation of the binding capabilities of candidate aptamers are presented. The techniques of SPR, EMSA, and dot blotting are evaluated and utilised in the appraisal of the novel aptamer sequences.

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List of Figures

List of Tables

List of Abbreviations

Ab	Antibody
AML	Acute Myeloid Leukaemia
ATP	Adenosine-5'-triphosphate
BALL	B-cell Acute Lymphoblastic Leukaemia
BNCT	Boron Neutron Capture Therapy
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
CE-SELEX	Capillary Electrophoresis SELEX
Da	Dalton
DNA	Deoxyribonucleic Acid
DPI	Dual Polarisation Interfereometry
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
(e)GFP	(enhanced) Green Fluorescent Protein
ELISA	Enzyme-Linked ImmunoSorbent Assays
ELONA	Enzyme-Linked Oligonucleotide Assay
EMSA	Electrophoretic Mobility Shift Assay
bFGF	Fibroblast Growth Factor
bFGF155	Human Fibroblast Growth Factor 155
FRET	Fluorescence resonance energy transfer
GELEX	Gel Electrophoresis SELEX
GST	Glutathione-S-Transferase
HBA	HsdR Binding Aptamer
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HRV143C	Human Rhinovirus 14 3C
HsdM	Host Specificity Determinant Modification
HsdS	Host Specificity Determinant Specificity
HsdR	Host Specificity Determinant Restriction
IPTG	Isopropyl-β-D-thio-galactopyranoside
IgG	Immunoglobulin G
LB	Luria Broth
Kd	Dissociation Constant
MRE	Molecular Recognition Element
NECEEM	Non-equilibrium Capillary Electrophoresis of Equilibrium Mixtures
NHS	N-Hydroxy-Succinimide
NT	Nitrotyrosine
OLR	Overlapping region
PAGE	Poly Acrylamide Gel Electrophoresis
PBA	HRV143C binding aptamer
PBS	Phosphate Buffered Saline
QCM	Quartz Crystal Microbalance
RM	Restriction Modification
RNA	Ribonucleic Acid
RU	Response Units
SDS	Sodium Dodecyl Sulphate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment

SPR	Surface Plasmon Resonance
ssDNA	Single Stranded Deoxyribonucleic Acid
STE	Sodium Tris EDTA
TAE	Tris Acetate EDTA
T-ALL	T-cell acute lymphoblastic leukaemia
TBA	Thrombin Binding Aptamer
TCR	T Cell Receptor
tRNA	Transfer Ribonucleic Acid
VEGF	Vascular Endothelial Growth Factor

1 Introduction:

1.1 Aptamers

Aptamers coming from the Latin "*apta*" meaning to fit, and "*mer*" meaning many, are short oligonucleotides ranging from 20-90 base pairs in length. Usually derived using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodologies, aptamers are seen to be a synthetic alternative to antibodies due to their high specificity and high affinity for their target (Stoltenburg *et al.*, 2007). Aptamers have specific advantages over antibodies because of their reduced production costs, ease of synthesis, and the fact that they do not require an organism for their production.

Considerable work has been undertaken within the two decades since aptamers and their potential were first identified. Aptamer technology can be separated into two groups, therapeutic and diagnostic, the aim of therapeutic aptamers can be seen as the use of aptamers as inhibitors, catalysts and mimics of enzymes and other molecules in a similar manner to many drugs. Currently the only aptamer to be approved (in the U.S.A) for use as a drug is *Macugen®* (Doggrell 2005), which is a treatment for wet age-related macular degeneration and this aptamer inhibits Vascular Endothelial Growth Factor (VEGF). The approval of this aptamer, and the success of treatment using this aptamer, is encouraging with regard to the potential use of aptamers in treatment of cancer and other diseases, as aptamers can now provide a new series of potential treatments. Diagnostic uses of aptamers primarily focus on the use of aptamers as molecular recognition elements within a larger biosensing system (Figure 1), using the aptamers as probes for target molecules in a similar way to antibody technologies (Jayasena, 1999).

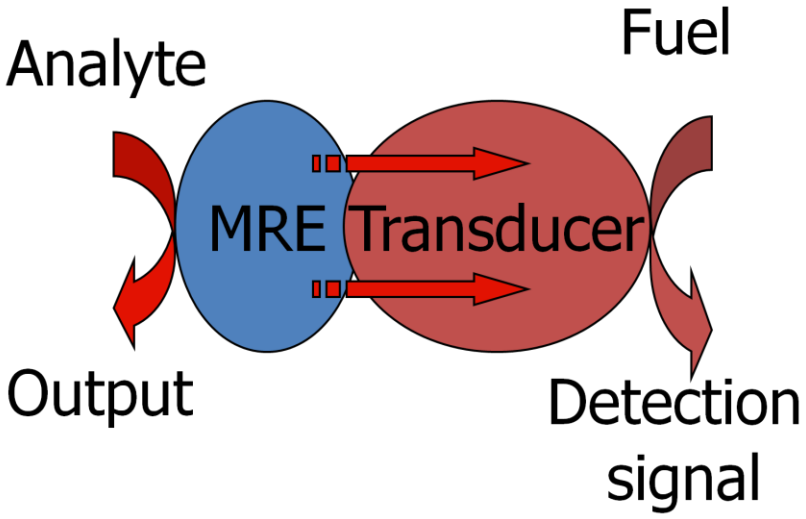


Figure 1 Schematic diagram of a biosensor. Cartoon of a generic biosensing system showing information being passed from analyte through to signal. This highlights the role of the Molecular Recognition Element (e.g. an antibody) within the biosensor acting as a probe for the analyte before the signal is passed on to the transducer (e.g. a fluorescent molecule) for detection.

Recent studies have investigated the use of aptamers as both the MRE and the reporter element by using chemical modifications of the oligonucleotides such as attachment of fluorophores, or the inclusion of modified nucleotides (Latham, Johnson et al. 1994; Li, Cao et al. 2008). The full breadth of possibilities of recognition for aptamers are still to be fully realised as aptamers are found to an increasing number of target molecules for example; small molecules, proteins, co-factors, even entire cells have aptamers evolved against them (Stoltenberg *et al.* 2007).

1.1.1 Standard SELEX

Systematic Evolution of Ligands using EXponential evolution (SELEX) was devised by (Tuerk & Gold, 1990) in 1990. A starting pool of oligonucleotides with random sequences, traditionally flanked by constant Polymerase Chain Reaction (PCR) primer regions, for the initiation of polymerisation, is introduced to a chosen, immobilised target molecule. Unbound sequences are washed away, whilst bound molecules are later eluted and amplified using PCR. The amplified library is then reintroduced to the target and the whole process is repeated. This repeated process replicates evolution in a rapid, microcosmic way.

The random library consists of single stranded oligonucleotides, typically 30-100 bases in length with a central section of random sequence. The size of the random library is determined to be 4^n (where ^{n} is the length of random sequence in bases) and tend to consist

of 10^{12} to 10^{14} different sequence (Stoltenburg et al., 2007). This library is then pre-screened against any part of the procedure that could produce a false positive, such as the media to which the target is bound, or part of the target which anchors the target, such as a chemical linker. Once the library has progressed through pre-screening it can be introduced to the objective for the SELEX process. Classically the target is bound to a matrix through a tag or linker (Stoltenburg et al., 2007) and the library of random sequences is screened for binding to the target by washing.. This process is repeated for 10-13 rounds of selection and amplification with the final library being isolated in a DNA cloning vector and sequenced with the goal of arriving at a consensus sequence. The consensus sequence is the final outcome of a successful SELEX selection procedure, detailed within the sequence would be any motifs or patterns that give the highest affinity and specificity for an aptamer to the target of interest.

1.2 DNA vs. RNA aptamers

Due to the definitive chemical difference between RNA and DNA, namely the 2'- hydroxyl group, which DNA lacks (Figure 2), DNA and RNA aptamers can form entirely different secondary structures to identical targets. The hydroxyl group found on RNA, provides a more reactive group when folding into secondary structures allowing the formation of hairpins, "kissing-loops" and pseudoknots. Multiple SELEX strategies for the RNA-based, ATP aptamer have been undertaken and the ç-loop (Sazani *et al.*, 2004) structure shown in Figure 15 has repeatedly been identified from a number of studies (Huiaenga(1995), Huang(2003), Feigon *et al.*(1996)). If, however, an RNA aptamer is converted to an identical DNA aptamer (with thymine, substituted for uracil) a reduced level of binding is observed (Lato *et al.*, 2002). The same can be said for the inverse situation, and this is the case for the majority of aptamers save only one reported instance for riboflavin (Lauhon & Szostak, 1995).

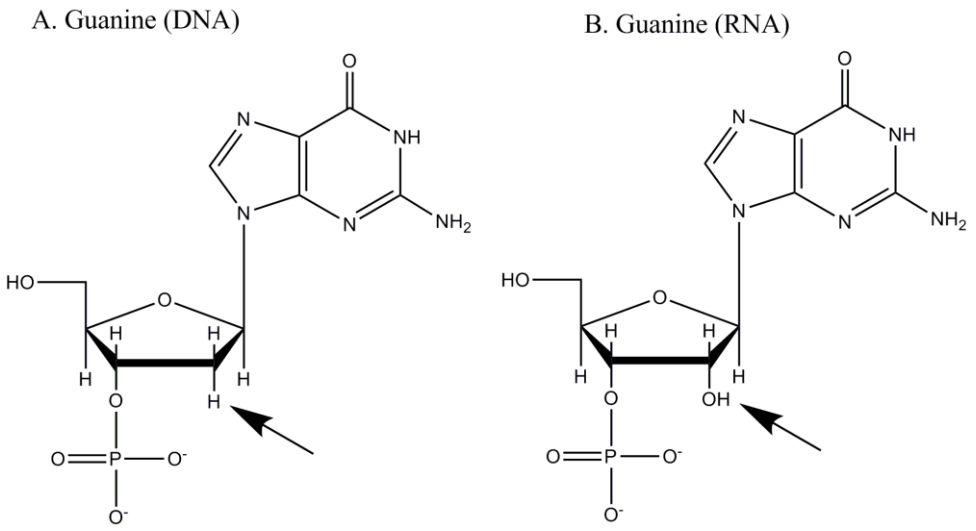


Figure 2 Nucleotide differences between DNA and RNA. Comparison of guanine in RNA and DNA form, highlighting the difference between the 2'-hyrdoxyl group lacking in DNA (A) and present in RNA (B), this is due to the C-OH bond found on RNA being more chemically reactive than the C-H bond found in DNA.

DNA can be considered a reasonably stable biomolecule, resistant to degradation over a range of different pH values and temperatures and it is because of this stability that DNA is so useful as a means for storing genetic material. Yet, it is RNA, which is repeatedly cited as the basis of all life - the idea of the "RNA world" was first discussed in the 1960s by Sol Speigelman (Spiegelman & Haruna, 1966), and the inherent ability of RNA to readily adopt secondary structures seems to have been the ideal catalyst for life and the cornerstone of genetic inheritance (Lam & Joyce, 2009). RNA is susceptible to degradation from environmental factors such as unfavourable pH and nucleases which can be attributed to the hydroxyl group found on RNA being more chemically promiscuous. The addition of the 2'-OH also improves stability by increasing the melting temperature and locking an RNA duplex into a more compact A-form helix compared to DNA's B-form helix.

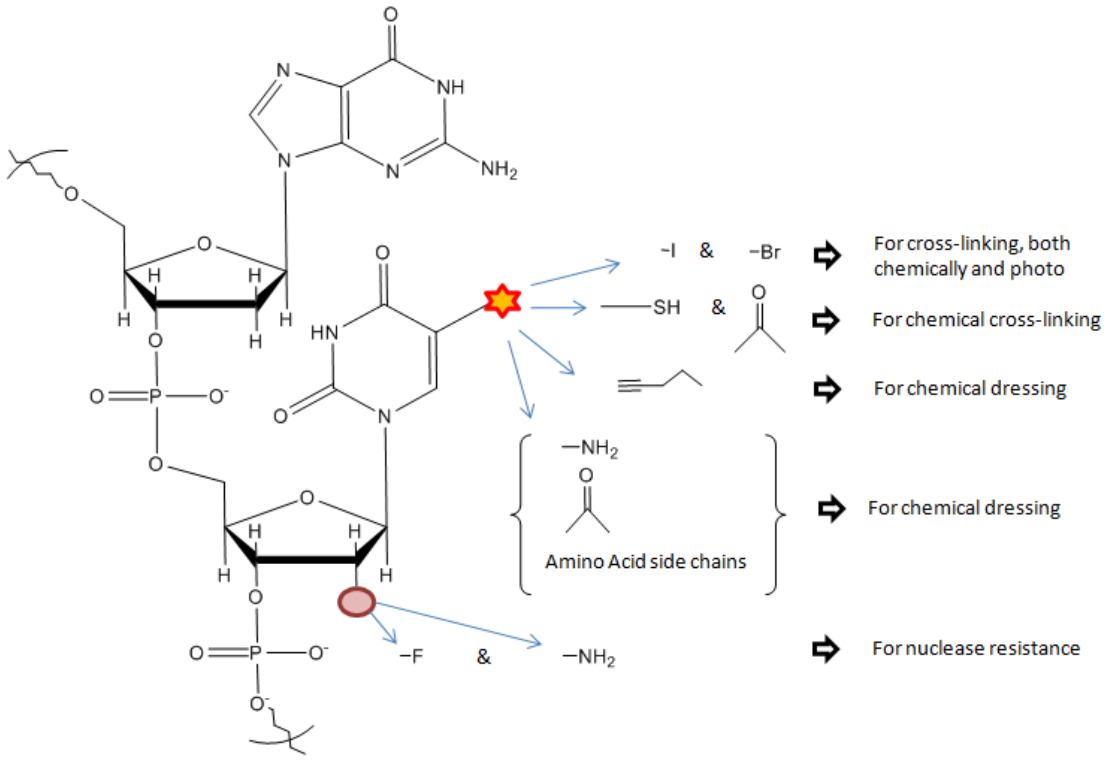
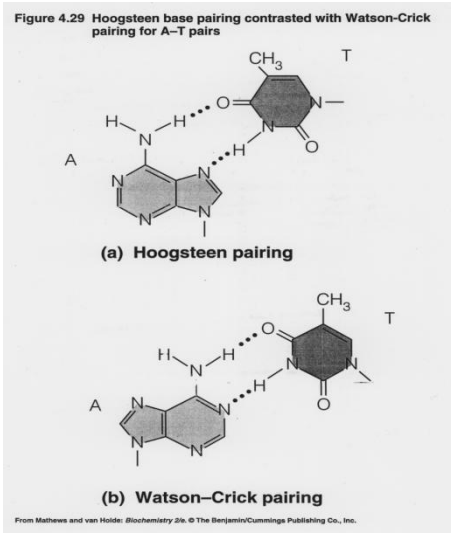


Figure 3 Nucleotide modification used in SELEX. Possible modifications on an oligonucleotide strand to generate modified oligonucleotide libraries for the SELEX process. Modification at the 2'- position (Circle) of the sugar confers nuclease stability, whereas various modifications at the C-5 position (Star) of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets. Modifications indicated by the arrows have represent some examples of the potential substitutions that could be used for the SELEX process.(Jayasena, 1999)

1.3 Secondary Structures

Aptamer binding relies on the bases of single stranded DNA, or RNA, forming conventional base pairs (Watson & Crick, 1953), Hoogsteen base pairing (Figure 4), base stacking between the pi bonds of the aromatic groups of the bases and the formation of secondary structures (Hermann & Patel, 2000). The three dimensional shape of the aptamer confers the specificity and affinity of binding to the target molecule. DNA secondary structures are less readily formed than the RNA counterparts because of weaker stacking energies (Chan *et al.* 1966), and the less reactive C-H bond found in the ribose of DNA compared to the C-OH counterpart of the 2'- carbon of RNA (Figure 2). The RNA pseudoknot (Figure 8) secondary structure is exclusive to RNA; whereas, the DNA G quadraduplex (Figure 7) tends to form within single, double and even multiple strands of DNA.

A) 

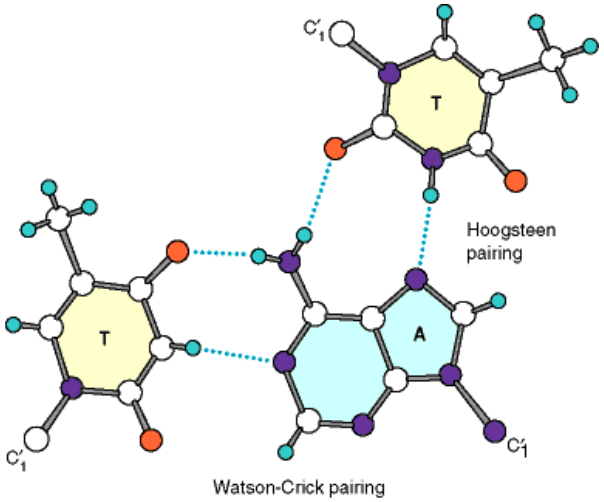
B) 

Figure 4 Diagram of Hoogsteen base pairing. A) Hoogsteen pairing is an alternative method for base pairing between adenosine and thymine residues with hydrogen bonding occurring between the N7 nitrogen of the adenosine rather than the N1. B) The alternative binding method provides the platform for impromptu triplex DNA formation (Matthews C., 1990).

1.3.1 Hairpins

DNA and RNA hairpins are the simplest oligonucleotide secondary structure, the formation of which are thermodynamically favourable due to the low energies required for base pair formation. A hairpin is formed when a length of ssDNA, or RNA, has regions of complementarity that flank an unstructured region, the complementary regions form base pairs resulting in a hairpin motif (Figure 5).

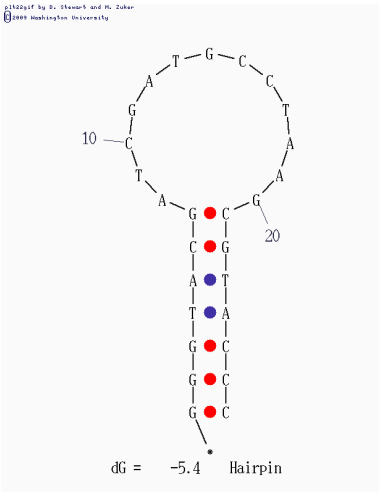


Figure 5 Diagram of a DNA hairpin. A diagram of a DNA hairpin in a short oligonucleotide, drawn using mfold DNA folding software (Zuker, 2003)

1.3.2 G-quadraduplex

Of the documented ssDNA secondary structure motifs the G-quadraduplex can be considered one of the most common within DNA aptamers (Gatto *et al.* 2009). As with the RNA pseudoknots, the G-quadraduplex has been found to be a biologically and physiologically relevant structure with particular relevance to the G-rich sequences found in eukaryotic telomeres. G-quadruplexes are higher-order DNA and RNA structures formed from G-rich sequences that are built around tetrads of hydrogen-bonded Hoogsteen paired guanine bases and often around a centrally located monovalent cation such as K^+ (Figure 6).

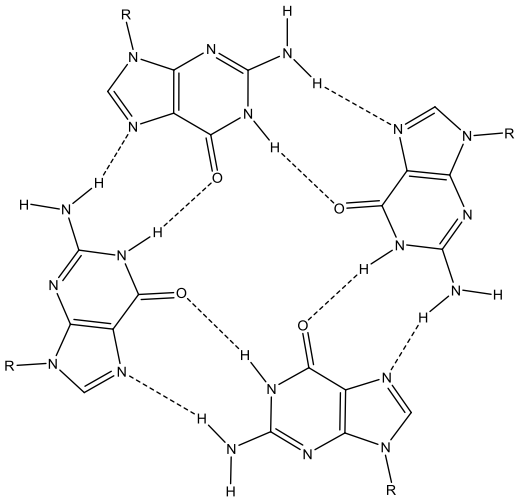


Figure 6 Base formation of a G-quartet. Four guanine residues forming a G-quadraduplex, the guanines can be on a single strand, or, two, three or four separate strands of DNA.

In addition to the proposed G-quadraduplex arrangement found in telomeres, non-telomeric genomic DNA, such as in nuclease-hypersensitive promoter regions, are also

thought to contain naturally occurring G-quadraduplexes forming from the G tetrad (Halder *et al.*,2005). The natural role and biological validation of these structures is now being explored, and there is particular interest in them as targets for therapeutic intervention (Burge *et al.*, 2006) The motif is now considered to be so important that there are drugs in development that target quadruplexes from particular genes (Ou. *et al*. 2008).

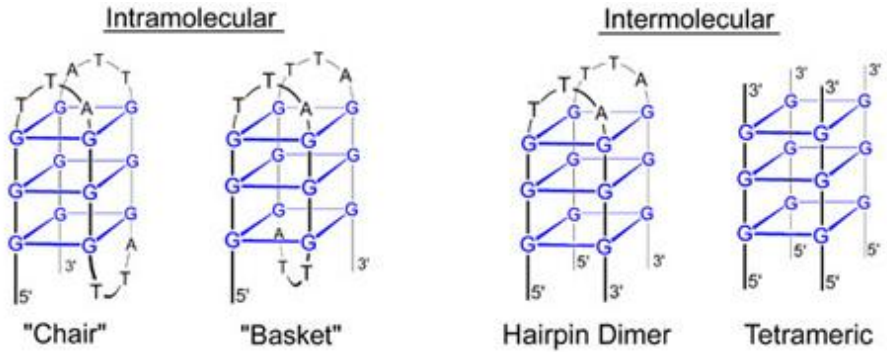


Figure 7 Comparison of intra- and intermolecular G-quadraduplexes. G-quadraduplexes can be assembled from single stranded DNA in an intramolecular (backfolded) fashion or intramolecular G-quadruplex topologies called 'chair' and 'basket' are shown Alternatively, up to four DNA strands can associate to form an intermolecular G-quadruplex. (Luedtke, 2009)

The biological relevance of the G-quadraduplex has become apparent following the analysis of full eukaryotic and prokaryotic genomes that have shown them to occur throughout the gemone (Todd *et al.*, 2005, Rawal *et al.*, 2006, Simonsson *et al.*, 1998).

1.3.3 RNA pseudoknot

The RNA pseudoknot is a secondary structure that forms when two stem loop structures occur and then interact with each other. Commonly found in nature, often within viral genomes (Pleij *et al.*, 1985), the RNA pseudoknot has been accepted to have widely varying functions within biological processes (Brierley *et al.*, 2007) ranging from the regulation of the initiation of protein synthesis by inducing ribosomal frame-shifting in many viruses (Shen & Tinoco, 1995, Barton *et al.*, 1999), to template recognition by viral replicase (Klovins & van Duin, 1999), or forming the catalytic core of various ribozymes (Rastogi *et al.*, 1996) (Ke *et al.*, 2004), the self-splicing introns (Adams *et al.*, 2004), and the core structural domain of human telomerase (Theimer *et al.*, 2005).

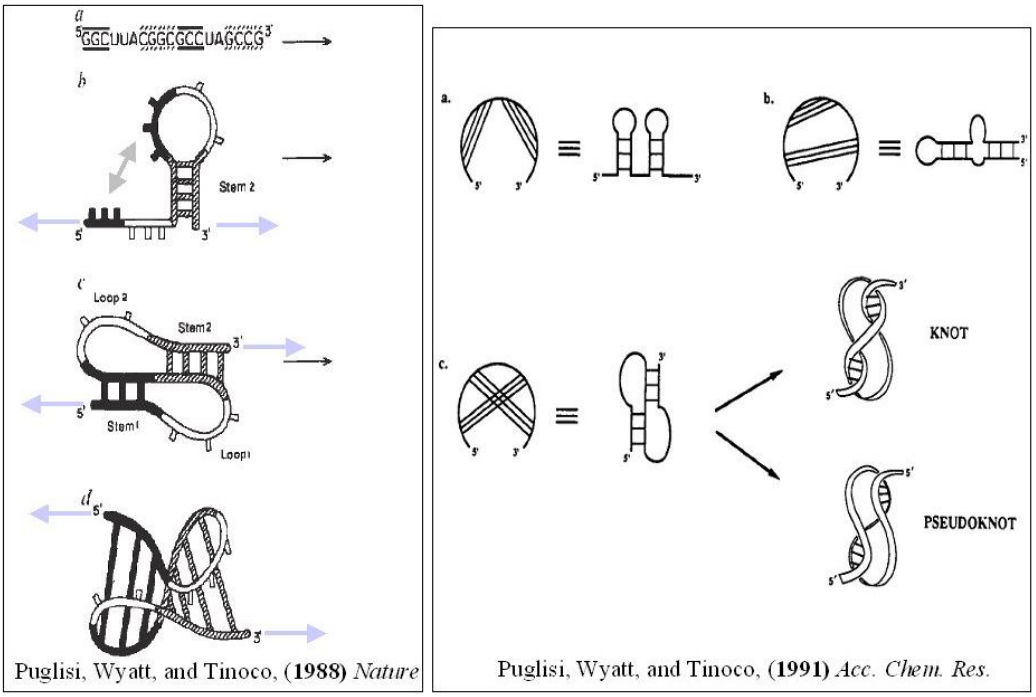


Figure 8 Formation of the RNA Pseudoknot. Cartoons detailing the formation of RNA pseudoknots through base to base interactions (Puglisi & Wyatt, 1995)

1.4 Aptamers vs. Antibodies

As mentioned above, aptamers are considered to be the nucleic acid equivalent of antibodies and also provide a reliable alternative to antibodies as molecular probes within a bio-sensor (Figure 1). Antibodies have been used extensively as molecular recognition elements since the 1970s, and their applications have become increasingly complex such as usage within Enzyme-Linked ImmunoSorbent Assays (ELISA) (Lequin, 2005), immunohistochemistry techniques(Buchwalow and Böcker, 2010), immunoprecipitation of biological molecules(Barrett *et al*. 1960), and cell differentiation in flow cytometry (Kamentsky, (1970), Dittrich & Göhde (1971)). Monoclonal antibodies – antibodies that recognise and bind a single epitope on the antigen - represent the pinnacle of antibody technology (Riechmann *et al.* 1988). This is where a specific clone for a known antibody has been isolated and reproduced using cell culture allowing large quantities of the antibody to be produced and the easier optimisation of downstream experiments(Kohler & Milstein, 1975, Schwaber & Cohen, 1973, Riechmann *et al.*, 1988). Antibodies, however, are not without their limitations, for example, when raising an antibody to an antigen, the target molecule typically needs to be introduced to a host animal, this procedure raises questions of ethics and also requires the target to draw an immunoresponse from the organism.

Additionally, if the target molecule is toxic to the animal it can prevent a sufficient response from the immune system, which can restrict the amount of antibodies retrieved. In contrast to antibodies, aptamers are, once isolated, easy to synthesise and do not require the use of any organism.

Due to the methods by which antibodies are derived and recovered there tends to be a large variation between each batch and this can be attributed to the varying levels in the organism's immune response, this results in immunoassays needing to be optimised for each batch of antibodies (Jayasena, 1999). When you compare this to nucleic acid synthesis where the methods of fabrication are constant and reliable and, as a result, high levels of aptamers can be produced quickly and with confidence of the accuracy of the chemistry. Modifications to aptamers are easily achieved using conventional chemistry methods by attaching functional groups during synthesis or by including modified nucleotides during synthesis. By this method fluorescein or biotin can be included either within or on a specific end of an aptamer with confidence (Agrawal *et al.*, 1986, Richardson & Gumport, 1983). These molecules confer a reporting ability onto aptamers that cannot be so easily achieved with antibodies without the use of a secondary antibody. As antibodies are organically derived probes they require close to physiological conditions for storage and use within other applications. This fragility also impacts on long term storage restricting the useful lifetime of the molecule, also once denatured the antibodies become irreversibly useless, in contrast aptamers can be stored, denatured and regenerated with relative ease. Aptamers will remain stable at room temperature and over a range of pH and salt concentrations, which, when compared to the more delicate antibody technologies, is a highly favourable situation, although alterations to the binding conditions can result in a loss of binding (Klussmann, 2006).

Therefore, aptamers can be seen as a viable alternative to antibodies and within this study the aim was to show the ease of isolation of aptamers in order to supplement and improve on antibodies as molecular recognition experiments within specific biosensor systems.

1.5 Aptamers as therapeutics

As aptamers have been shown to exhibit a high binding affinity to the target and a selectivity that is usually associated with antibodies, there are a growing number of

instances of aptamers being used as treatments rather than just drug delivery tools. For example, the major goal for boranophosphate nucleotide aptamers would be their use as a suitable delivery method of boron for use within Boron Neutron Capture Therapy (BNCT, Figure 3,Lato et al., 2002) providing a low toxicity cancer treatment as an alternative to conventional chemotherapy. The boron-containing aptamers are selected towards tumour-forming cancer cells using Cell-SELEX (Shangguan *et al.*, 2007, Shangguan *et al.*, 2006) and only the species with the highest affinity specificity are chosen. Due to their innate resistance to blood borne nucleases, the boranophosphate nucleotide containing aptamers have an increased half-life *in vivo* (Porter *et al.*, 1997). The affected area is then irradiated with neutrons which are, in turn, accepted by the ^{10}B converting it to ^{11}B. The ^{11}B then releases radiation in the form of ^{7}Li and ^{1}H nuclei and ^{4}alpha radiation killing all tissue within a 5-9 µm radius of the boron isotope. The inclusion of boron-containing 5'-α-borano G and U within an aptamer using wild-type T7 RNA polymerase proved successful, with multiple anti-ATP aptamers having been identified that made use of the presence of boranophosphate linkages to bind the target (Lato et al., 2002). The use of a well–characterised and understood aptamer, in this case the anti-ATP aptamer, to test a new aptamer technology can be reasoned because the binding of the aptamer to the target can be assumed to be consistent with the reported affinity. Additionally, comparisons can be drawn with regards to any difference in binding that can be elucidated from the study.

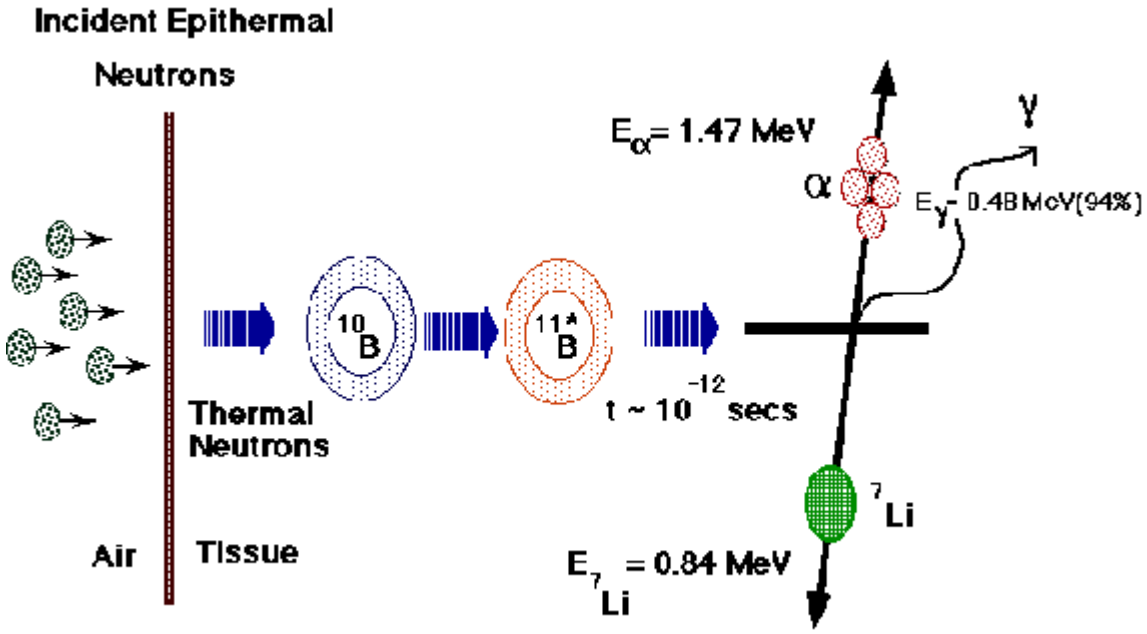


Figure 9 Schematic Diagram of BNCT. The firing of a neutron beam will convert ^{10}Boron to ^{11}Boron causing the Boron to divide into ^{7}Lithium and the release of ^{4}alpha particle radiation causing a local ionization over a region of 1 µm diameter. This causes localised cell death within an area of around a cell's width of the Boron nucleus.

1.6 Alternative SELEX techniques

Due to the repetitive nature of the SELEX methodology and the length of time that standard SELEX can take to successfully amplify an aptamer, alternative methods to produce useful aptamers have been sought. SELEX, in essence, is the selection of prospective ligands based on the separation of target-bound and unbound species, so, there is large potential for novel methods of partition and augmentation of the procedure. Detailed below are a number of alternative SELEX techniques that differ from standard SELEX and the benefits and detractors of each system.

1.6.1 Capillary Electrophoresis SELEX

Capillary Electrophoresis SELEX (CE-SELEX) or Non-equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM)(Berezovski *et al.*, 2003) is rapidly becoming the most reliable and efficient way to develop aptamers from the oligonucleotide pool, this method involves immobilising the target to the wall of a capillary tube of the Capillary Electrophoresis device using covalent chemistry, and passing the aptamer library over it. Some species within the library are retarded by interactions with the target and those with the lowest dissociation constant are held longest.

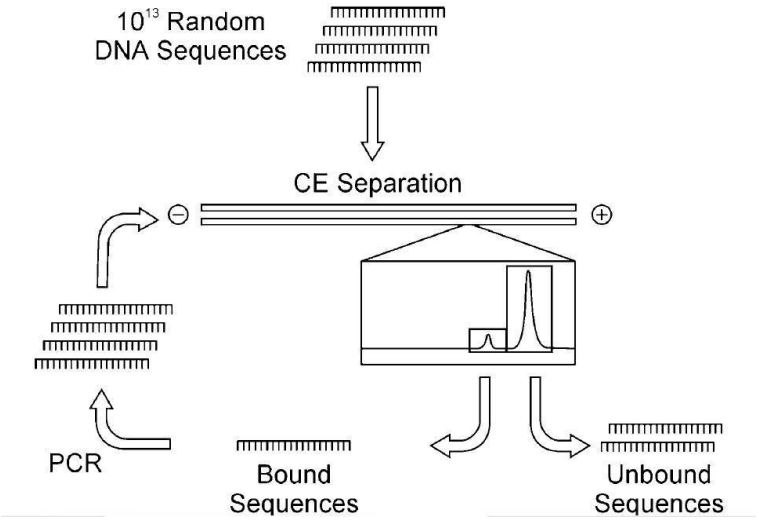


Figure 10 Schematic of CE-SELEX. A library of ssDNA molecules is incubated with target molecule. Capillary electrophoresis is used to isolate bound sequences. Binding oligonucleotides are PCR amplified and purified giving an enriched ssDNA pool, suitable for further rounds of selection. High affinity aptamers are typically obtained after two to four rounds of selection. Figure taken from (Mosing et al., 2005)

Derived from a oligonucleotide purification technique (Cohen *et al.*, 1988), CE-SELEX was first described in 2002 (Clark & Remcho, 2002) and relies on the refinement of an aptamer library following repeated rounds of SELEX using capillary electrophoresis to ensure the separation of bound and unbound species. Due to the advanced separation efficiency the number of rounds required to isolate aptamers with high binding efficiencies, can be reduced to 2–4, after this, generally no further improvement in binding efficiency is usually observed (Mendonsa & Bowser, 2004). CE-SELEX has been successfully employed to raise aptamers to three large proteins: Age (Mendonsa & Bowser, 2004), farnesyltransferase (Berezovski *et al.*, 2005), and HIV-1 Reverse Transcriptase (Mosing et al., 2005) and also to two smaller molecules: Ricin toxin (Tang *et al.*, 2006), Neuropeptide Y (Mendonsa & Bowser, 2005) and most recently the anthrax toxin polypeptide (Cella *et al.*).

An apparent limitation associated with the CE-SELEX methodology is a reduced starting library, this is a result of the low sample volume, which is often “touted” as one of the benefits of the technique (Mosing et al., 2005). The reduced volume of approximately 50 µL restricts the number of species from the starting ~10^{13} library that can be introduced to the target, therefore, omitting many potentially binding species from selection.

1.6.2 Modified Oligonucleotide-SELEX

Mod-SELEX is SELEX technique that is undertaken with modified nucleotides; these include morpholinos as well as 2'- fluoro nucleotides (Keefe & Cload, 2008). The limiting factor involved in this form of SELEX is deemed to be the polymerase involved in the PCR amplification steps. The problem is whether the chosen polymerase will incorporate the modified nucleotides. There have been several studies to optimise the incorporation of modified nucleotides (Sousa & Padilla, 1995, Huang *et al.*, 1997, Fa *et al.*, 2004) by the existing widely used polymerases, such as *taq* polymerase or T7 RNA polymerase or, whether novel polymerases need to be identified that will integrate the alternative nucleotides (Chelliserrykattil & Ellington, 2004).

Aptamers constructed with modified nucleotides have one major advantage, complete, or partial, resilience to degradation by nucleases (Strehlitz *et al.* 2008). This in turn can increase the potential use of aptamers as therapeutics *in vivo*. If modifications to an aptamer which has been selected to target a protein present in blood can increase the half life of the aptamer, but the total residency time *in vivo* remains limited, then the use of an aptamer as a therapeutic reagent can be considered viable. In 2000, the first aptamer therapeutic was approved by the FDA in the USA. *Macugen®* is now commercially available as a drug therapy for macular degeneration. The aptamer in question targets a protein Vascular Endothelial Growth Factor VEGF-1 known to be instrumental in the progression of the degenerative disease (Mousa & Mousa).

Mod-SELEX pathways can be further categorised by the modified nucleotide that is incorporated into the oligonucleotide:

1.6.2.1 2'-Amino pyrimidines

The first successful attempt to substitute modified nucleotides, achieved by Lin *et al* (Li *et al.*, 2008), was an attempt to investigate the potential of aptamers as therapeutics, in this case a resistance to blood-borne nucleases. 2'-amino pyrimidines and 2'-hydroxyl purines were included in the starting library and throughout every subsequent amplification step giving rise to an aptamer that binds and inhibits fibroblast growth factor (bFGF) with high affinity (350pM) and is 1000 times more stable than RNA in human serum and urine. The use of 2'-amino pyrimidines however, is limited due to difficulties arising during the

chemical synthesis of the 2'-amino pyrimidines which restricts the amount of the nucleoside that can be produced per synthesis (Jellinek *et al.*, 1995)

1.6.2.2 2'-fluoro pyrimidines

The most successful attempt to incorporate modified nucleotides in order to prolong the *in vivo* half-life of an aptamer, and create the first therapeutic aptamer, involved the incorporation of 2'-fluoro pyrimidines into a well-described aptamer. Although not technically derived from mod-SELEX as the modified nucleotides were substituted for the wild-type hydroxyl pyrimidines as a post-SELEX optimisation, the target was Vascular Endothelial Growth Factor a protein involved in age-related macular degeneration. Another 2'-fluoro pyrimidine based aptamer that is currently undergoing clinical trials, is the anti-Factor IX aptamer RB006 (Keefe & Cload, 2008). This aptamer is intended to act as an anti-coagulant by interfering with the blood clotting cascade by means of binding and inhibiting the enzyme involved in coagulation of blood.

Higher yields of amplification products during Mod-SELEX, using the traditional SELEX method, can be achieved using 2'-fluoro pyrimidines and the Y639 mutant of T7 RNA polymerase for the PCR amplification stage suggesting that mod-SELEX can be undertaken with the same efficiency as conventional SELEX (Kopylov & Spiridonova, 2000). The Y639 mutant of T7 RNA polymerase (Kostyuk *et al.*, 1995) can incorporate both 2'-deoxy-nucleotide and non-deoxy nucleotides when synthesising oligonucleotides with no loss of specificity for the substrate as the reduced fidelity of the mutated enzyme allows the efficient incorporation of non native nucleotides.

1.6.2.3 2'-O-methyl pyrimidines

2'-O-methyl nucleotides are a naturally occurring modified nucleotide, which also help to confer nuclease resistance to aptamers during post-SELEX modifications whilst providing chemical stability. The 2'-O-methyl can occur as a post-transcriptional modification to RNA with an occurrence of around 100 per ribosome (Burmeister *et al.*, 2005) and studies using the modification have been successful by incorporating the nucleotide using the mutant Y639F/H784 T7 polymerase for PCR. In a study by Burmeister aptamers were successfully raised to VEGF including the 2'-O-methyl nucleotide post SELEX modification have found to have a dissociation constant of 2 nM (Burmeister *et al.*, 2006).

1.6.2.4 4'-Thio pyrimidines

In another approach for confering nuclease resistance to an aptamer, the 4'-oxygen atom of the nucleotide ribose ring was targeted as a possible site of modification. By substituting 4'-thio UTP and CTP into the SELEX library during selection for a modified thrombin binding aptamer, Kato *et al* reported a 50-fold resistance to RNase A-mediated degradation when compared to unmodified RNA, whilst retaining a dissociation constant of 5 nM for the aptamer-target (Kato *et al.*, 2005).

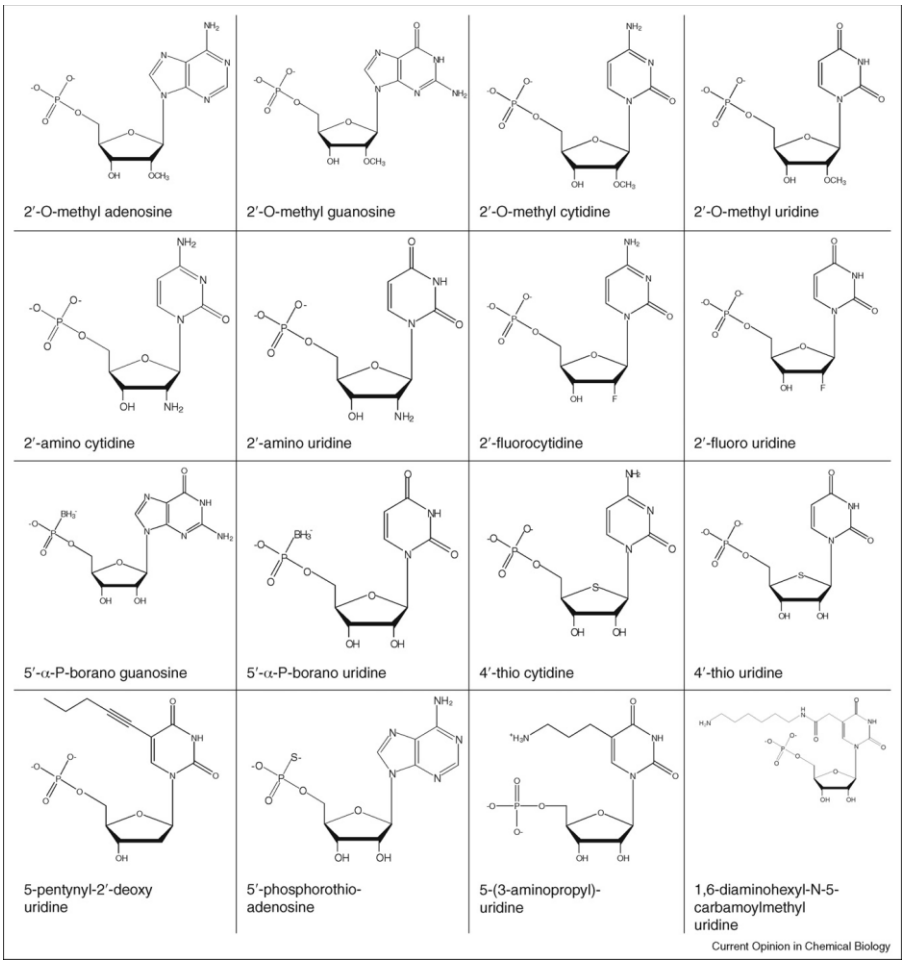


Figure 11 Diagram of modified nucleotide bases available. Grid detailing some of the modified nucleotides and nucleosides available for nucleic acid variation.

1.6.3 Toggle SELEX

Occasionally a situation arises where it is beneficial for an aptamer to have specificity towards two similar, but distinctly different targets, two isoforms of a protein for example. In these cases toggle-SELEX (White *et al.*, 2001) can be utilised. It consists of conventional

SELEX rounds of screening and amplification, but, instead of screening against one target consistently the library is introduced to the variants in alternating rounds of screening step (Figure 6).

The major benefit of toggle-SELEX is that the resulting aptamers will tend to bind the closely related regions that will exist in both two related targets. In the first reported example of "toggle" SELEX (White et al., 2001) the aptamer library was screened against human and porcine thrombin and produced RNA "toggle" aptamers that bound human thrombin with dissociation constants from 1 to 4 nM and porcine thrombin with a dissociation constants for the screened library all less than 1 nM whilst maintaining their ability to inhibit the clotting activity of both human and pig blood clotting Factor IIa.

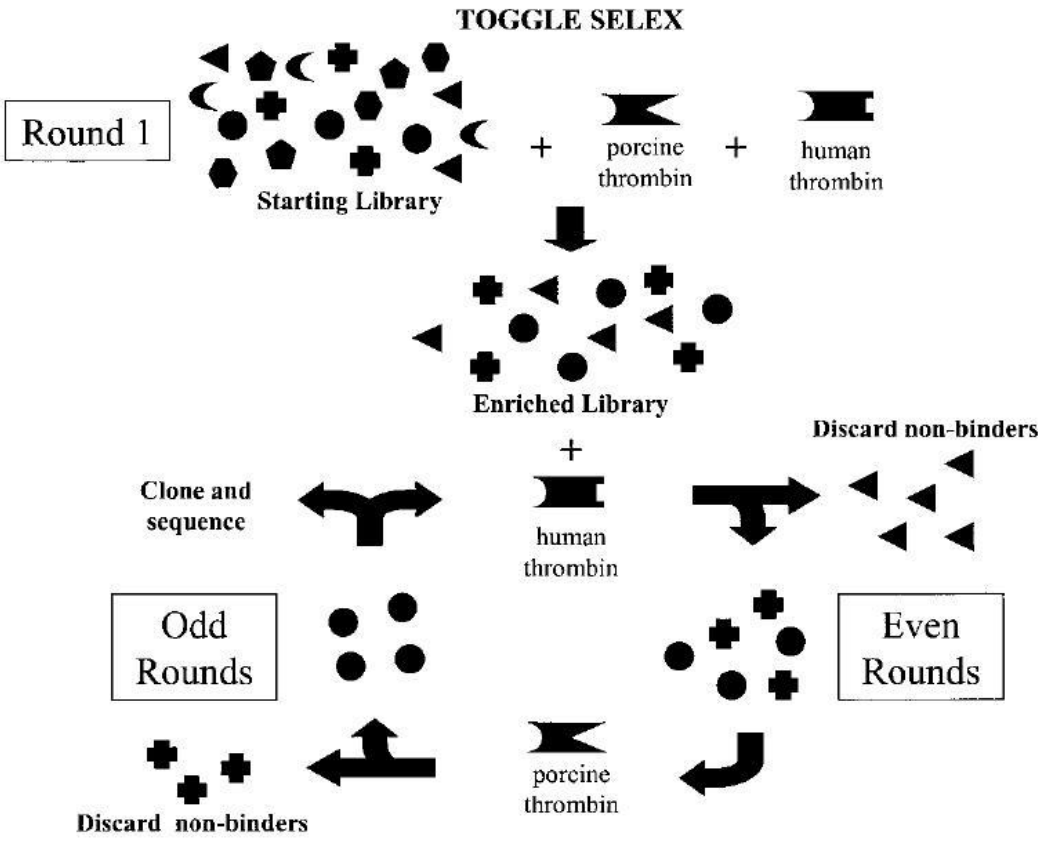


Figure 12 Schematic diagram of "toggle" SELEX methodology. Schematic diagram of the toggle SELEX process. Libraries are exposed to two related targets in alternative rounds of selection, enriching a library towards the two antigens (White, Rusconi et al. 2001).

1.6.4 Photo SELEX

Aptamer binding relies solely on the shape which the potential aptamer adopts when folded in predefined conditions, these conditions however, are likely to be optimised so that the target protein remains in its native state. Although maintaining these conditions may preserve the structure of the target molecule, it may not be conducive to the best possible binding by the aptamer because the pH or solvent concentrations may prevent a potential aptamer from forming a suitable secondary structure required for the tightest binding. An interesting mechanism to overcome this problem is to photochemically crosslink the potential aptamer(s) to the protein of interest and as a result any aptamers that have weak interactions can be covalently linked to the protein of interest (Golden *et al.*, 2000), which allows isolation of these aptamers. The process of cross-linking requires the incorporation of a modified nucleotide into the potential aptamers during the amplification steps that will absorb light and are excited sufficiently to form a bond with the aromatic and sulphur bearing amino acid residues found in the target protein. Golden *et al.* (2000) described the use of 5-bromo-2'-deoxyuridine (BrdU) to replace thymidine as BrdU absorbs light in the 310 nm range where the native chromophores of nucleic acids and proteins do not absorb, or absorb only weakly. This absorption of light at 308 nm results in cross linking by photo-coupling of BrdU nucleotides that are located in a reasonable proximity to the aptamer (Dietz & Koch, 1987, Dietz & Koch, 1989).

Using this method aptamers have been produced that show an unprecedented affinity for the target human fibroblast growth factor 155 (bFGF155). The two aptamers 06.50 and 06.15, derived in this study, were found to have dissociation constants of ≈16 pM and ≈560 pM respectively (Golden et al., 2000), which compare very favourably to conventionally made aptamers for the same targets that show interaction dissociation constants of for the aptamers 06.15 and 06.50 that range in the tens of nM.

1.6.5 Cassette Expression SELEX

The use of aptamers as therapeutic agents still has a significant barrier with regard to the delivery of aptamers *in vivo* to the site of action; the majority of organisms have methods for ensuring that foreign DNA and RNA are quickly broken down and destroyed. Cassette Expression SELEX avoids the need to deliver the foreign aptamer into the cell by deceiving the organism into fabricating the nucleic acid sequence itself *in vivo*.

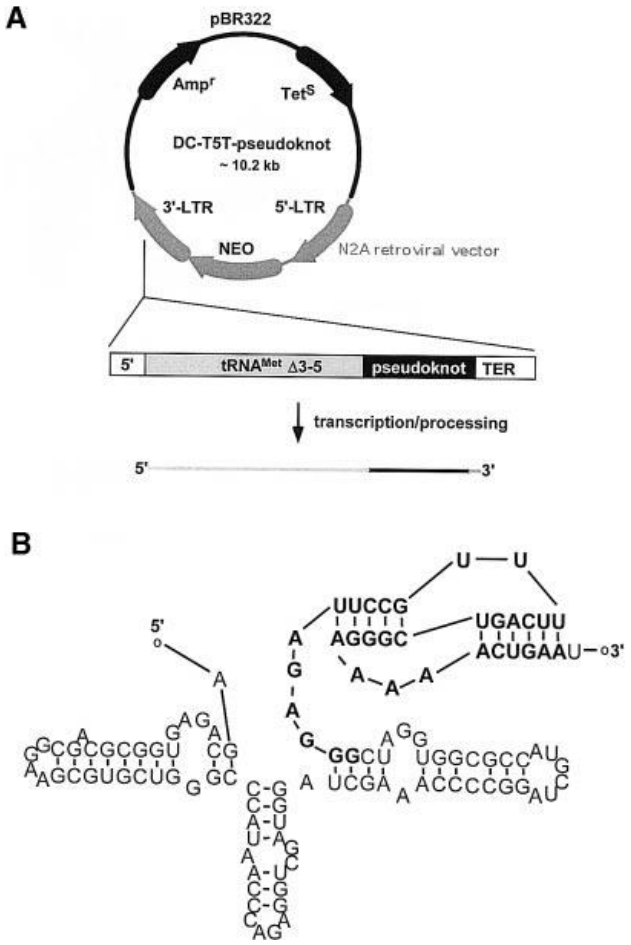


Figure 13 Construction of the expression cassette vector. An oligonucleotide containing the pseudoknot sequence was cloned downstream of the tRNA gene and upstream of the polymerase III termination signal generating the DCT5T-pseudoknot vector. (B) Secondary structure prediction of the pseudoknot motif within the chimeric tRNA-pseudoknot RNA transcript. The pseudoknot part is shown in bold letters. The additional base at the 3'-end of the transcript arises from the termination signal (Chaloin et al., 2002).

The process involves the use of a retroviral vector (Chaloin et al., 2002) created from a chimera that was produced by transiently expressing the molecule in eukaryotic cells as a polymerase III-driven chimeric gene that consisted of the human initiator $tRNA_{Met}$ ($tRNA_{Meti}$) sequence and the 33 nt pseudoknot sequence. This method inserts the aptamer sequence into the flanking regions of the viral genome to produce the chimeric $tRNA_{Met}$/RNA aptamer that binds and inhibits the HIV type I reverse transcriptase. This technique provided a 75% inhibition of HIV particle release when co-transfected with HIV-1 DNA in human 293T cells. Another report of the use of this technique (Martell *et al.*, 2002) inserted an aptamer that inhibits a family of transcription factors. These transcription factors provide a crucial role in the process of cell proliferation. The aptamer, called the E2F aptamer, was placed into a

tRNA expression cassette and applied to the virus *in vitro*. This study demonstrated high levels of chimeric tRNA that bound its target protein E2F1 with high affinity and in addition provided inhibition of functional transactivation by up to 80%.

1.6.6 Primer-free SELEX

An essential part of SELEX and, by extension, aptamer isolation is amplification of DNA or RNA, between rounds of screening, and due to the nature of amplification using PCR the use of fixed primer regions is fundamental (Yang *et al.*, 2007). The problems arising from this is that after selection these regions need to be removed as the constant regions can interfere with the elucidation of the best binding sequences by providing false positives and preventing the random sequence from forming the secondary structures required for efficient binding (Pan *et al.*, 2008). By including an additional cleavage step to remove the priming sections of the library before the screening part of the SELEX process the interference by constant regions is minimised.

1.6.7 Cell-SELEX

Cell-SELEX or complex target SELEX (Morris *et al.*, 1998) focuses on whole cell targets for selection and tends to identify aptamers to surface bound membrane proteins, but it does not require the potential aptamers to exclusively bind to the outer membrane of the cell. In fact, the first theory advanced suggested that the random DNA library was absorbed into the cell and the aptamers bound to cell-specific targets within the cell (Daniels *et al.*, 2003). In one particular case (Daniels et al., 2003),the selection was performed by creating a monolayer of U251 glioblastoma cells, a series of washing steps, then harvesting of the cells before a recovery of the DNA using phenol:chloroform extraction and ethanol precipitation. After 21 rounds of repeated screening and washing the library was cloned and 163 clones of possible aptamers were obtained. After computer based analysis (method not reported) of the motifs and the recurring clonal dominance of some species, one aptamer sequence was decided upon as the consensus sequence, (CCCAGAGGGAAGACTTTAGGTTCGGTTCACGTCC) (Daniels et al., 2003).

The ability to selectively bind cell surfaces has a huge potential for the future application of aptamers, both therapeutic and analytical. An aptamer that selectively binds a certain type

of tumour cell could be used both as a potential guide for drug targeting, or, in line with modern techniques, as a probe for affinity-based sorting and tagging (Daniels et al., 2003).

In order for the application of Cell-SELEX to be successful in this area, attention needs to be focussed on whether the target cells can be differentiated following up-regulation of specific proteins expressed on the cell surface. It is the displayed and accessible membrane proteins of the cell surface that enable the technique of Cell-SELEX (Fang 2009) although, the proteins of interest may only be elucidated after further investigation using the resulting aptamer as probes in an affinity purification procedure.

Studies to discover aptamers selected via this method tend toward cancerous and healthy cell differentiation with aptamers being selected to several cell types such as small cell (Chen *et al.*, 2008) and non-small cell lung cancer (Tang *et al.*, 2007b), liver cancer (Shangguan *et al.*, 2008b) and lymphocytic and myeloid leukaemia (Shangguan et al., 2006).

1.6.8 Surface binding

The generic biosensor largely requires the interaction of a ligand with a known binding partner that has been immobilised on a surface in order for detection to occur. Electrochemical aptasensors (Xu 2009) work in this way for example, along with Surface Plasmon resonance (Jost *et al.* 1991) and the quartz crystal microbalance (QCM) (Hianik 2005). As a result some attention must be paid to the methods available for tethering a biologically active molecule to a surface.

Streptavidin is a 60,000 Da tetrameric biotin-binding protein that was originally isolated from *Streptomyces avidini* (Chaiet & Wolf, 1964, , 1998). It consists of four subunits which are capable of binding four D-biotin molecules. D-biotin has a molecular weight of 244 Da and binds tightly to streptavidin with a dissociation constant of $K_d=10^{-15}$ mol dm^{-3} (Green, 1975). The strong interaction between D-biotin and streptavidin has been utilised in a wide range of technologies ranging from affinity chromatography to biochemical assays (Wilchek & Bayer, 1990)

An alternative to the use of a biological methodology to immobilise a biomolecule to a surface is the use of amine coupling (Fischer 2010). This method works by the activation of a surface that will result in ester bonds forming primarily with the lysines and the α-amino

groups of proteins (Aslam and Dent 1999). A common biological method for the surface attachment of proteins when orientation is not crucial is the use of N-hydroxy-succinimide (NHS) esters, these chemical bonds have been found to be moderately reactive toward amines, with high selectivity toward aliphatic amines(Smyth *et al.* 1964). The use of NHS esters is desirable for biological methodologies because the reaction rate with aromatic amines, alcohols, phenols, and histidine is relatively low(Smith 1993), this allows the selection of molecules that adsorb to the surface under known conditions.

1.7 Peptide Aptamers

Given the success of RNA and DNA aptamers as tools for molecular recognition and the detection of a wide range of biomolecules, other polymeric biological molecules, that have an ability to form distinct three dimensional structures, have been investigated with the aim of providing alternative technologies to the established and well characterised use of nucleic acids (Gold *et al.* 2010). The leading alternative to nucleic acid aptamers are peptide aptamers (Crawford *et al.* 2003). Although derived by the more laborious technique of using a microarray of potential sequences, peptide aptamers are distinct from traditional peptide binding sequences as only through the formation of the 3D secondary structure is binding of the peptide to the target facilitated (Colas 2008). Peptide aptamers were designed using the IgG antibody as a model, the aptamer consists of a "scaffold" and a variable region inserted onto it and although the synthetic peptide aptamer is not as ordered (Figure 14) as the IgG molecule, the similarity between the two remains (Baines & Colas, 2006). However, the major benefits of a peptide aptamer over IgG is there is no requirement for disulphide bonds required to enable binding (Colas 2008) and the relative compactness of the peptide aptamer (Colas, 2008). Additionally the scaffold can be used to confer a possible reporting or probing ability onto the peptide aptamer, historically the most common scaffold protein was thioredoxin, (LaVallie *et al.*, 1993), a fusion protein that allowed swift and reliable purification of the proteins. Other scaffolds for the delivery of the peptide aptamers have also been used, green fluorescent protein (GFP)(Abedi *et al.*, 1998), which confers an inherent fluorescent reporting ability to the molecule, Staphylococcal nuclease (Norman *et al.*, 1999) and Stefin A, a protease inhibitor (Woodman *et al.*, 2005) both of which provide biologically inert scaffolds that remain stable in physiological conditions in order to maintain the conformation of the peptide aptamer.

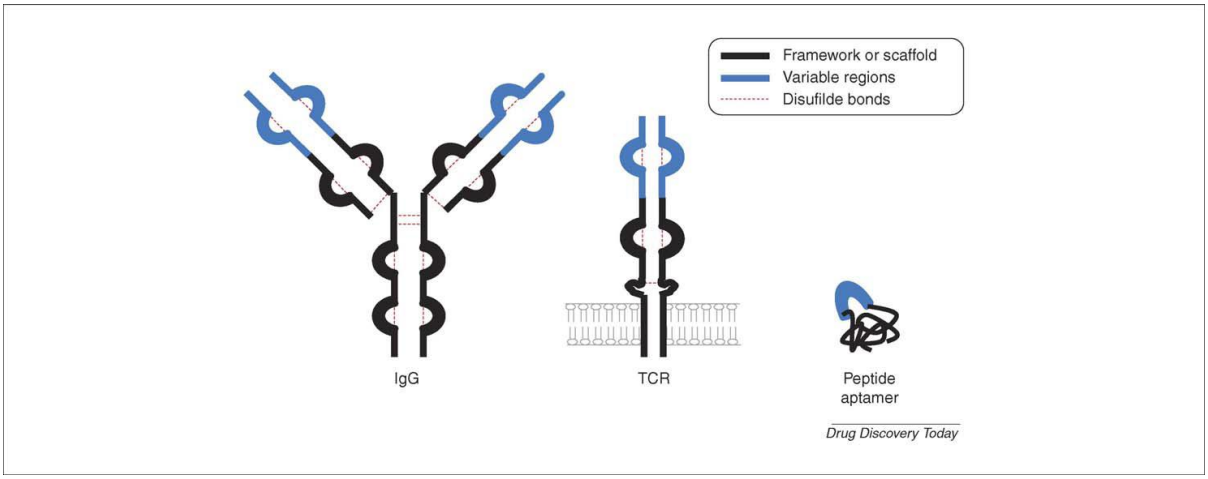


Figure 14 Peptide based recognition molecules. Three major classes of constrained recognition protein molecules are depicted: immunoglobulin G (IgG), T cell receptor (TCR) and peptide aptamer. Black lines represent constant framework or scaffold residues. Blue lines represent variable peptidic regions. Red dotted lines represent disulfide bonds. Major distinguishing features of peptide aptamers include the much smaller size and the absence of requirement of disulfide bonds to ensure structural constraint of the variable region, as opposed to IgG and T-Cell Receptor(TCR) molecules (Baines & Colas, 2006).

The method for peptide aptamer derivation relies on a microarray system, which can contain from 10 to 10^9 individual peptide probes in the microarray a number that is 2-4 orders of magnitude smaller than oligonucleotide random libraries (Colas *et al.* 1996). This inferred lack of complexity within the peptide starting library can be seen as a problem for the validity of the technique, but can be used to create structured libraries that focus on predicted motifs. Using the information that has been gathered from collections of reagents with peptide aptamers that have already been selected for and characterised and that are known to specifically bind proteins and their mutant isoforms, the structured libraries can lead to enhanced rates of peptide aptamer identification, greater specificity and greater affinity between the aptamer and target (Colas, 2008).

1.8 Previously described aptamers

In order to evaluate the usefulness of nucleic acid aptamers and compare the technology to an established and widely used system, there is a requirement to isolate aptamers to diverse targets. This process requires specific time and dedication due to the nature of SELEX and more specifically there remains no reliable high-throughput method for the production of novel aptamers. When the need for an aptamer to a target has been identified there must an intensive search of the literature in order to establish whether a

previous study has identified a consensus sequence or even a pool of sequences known to bind the target with a high specificity and affinity.

Due to the rapid growth of the field of aptamer research, it is difficult to estimate the number of targets to which aptamers have been described. A recent study provides an encouraging report of the reproducibility across different SELEX techniques, in this account three separate projects result in the production of consensus sequences to the 60 kDa protein streptavidin (Bing *et al.*). A comparison of the resultant consensus sequences displays a common motif of a hairpin loop structure with a characteristic bulge along the leading (5') strand of the formation.

The following section aims to identify and highlight previous investigations that have proven successful in the identification of novel aptamer sequences whilst providing a rationale as to why each target has been identified as such.

1.8.1 ATP-binding DNA and RNA aptamers

An early development in the establishment of aptamers as viable alternatives, if indeed superior, to antibodies was the discovery that aptamers also bound small molecules (less than 88 Da). Of the small molecules which aptamers have been shown to bind the most thoroughly researched is the adenosine-5'-triphosphate (ATP) binding aptamer. The first aptamer to be isolated that target ATP was an RNA aptamer, which was first isolated in 1993 (Sassanfar & Szostak, 1993) with the solution structure following in 1996 (Dieckmann *et al.*, 1996). A DNA-based ATP aptamer was first described in 1995 (Huizenga & Szostak, 1995). Although ATP was one of the first targets to have aptamers produced using both DNA and RNA, it was quickly found that each nucleic acid made use of a distinct consensus sequence that produced radically different secondary structures in order to bind the target. It was proposed by Huizenga and Szostak (Huizenga & Szostak, 1995) that the DNA aptamer formed a G-quadraduplex; whilst projected secondary structure models for the RNA-based ATP binding aptamer indicated an 11 nucleotide loop that encircled the ATP molecule. The 11 nucleotide loop has repeatedly been reported and is considered one of the most conserved and stable motifs found in aptamers isolated using SELEX (Figure 15) (Huizenga & Szostak, 1995).

An interesting ability of the RNA-based ATP aptamer is the ability to bind to several analogues of ATP, with the apparent target for the aptamer being more specifically the adenosine base of the molecule rather than the molecule as a whole. These aptamers show the ability to bind 3'-5' cAMP, ADP, dATP, 2'-O-MeA, 5'AMP, ITP and NAD, but a deficiency for binding other ATP analogs that lack the adenosine (Lato et al., 2002).

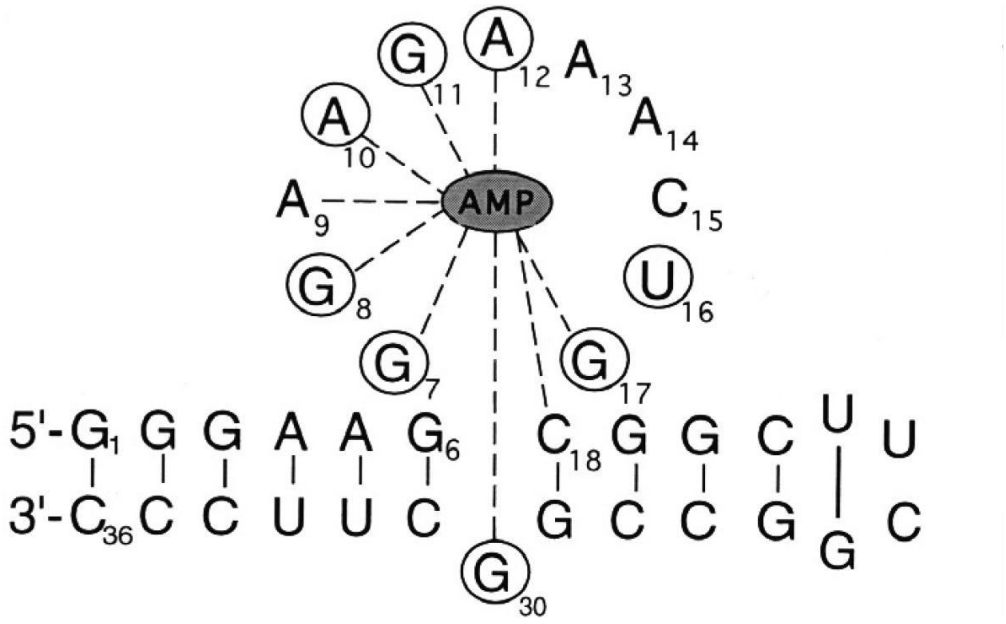


Figure 15 RNA anti-AMP aptamer. Proposed structure for the ATP/AMP binding RNA-based aptamer. The sequence was first reported in Sassanfar and Szostak (1993) and the circled nucleotides represent bases that were conserved in all of the sequences that were isolated. Diagram reproduced from (Dieckmann et al., 1996).

Further studies of the RNA-based ATP binding aptamer provided evidence that the highly conserved aptamer consensus sequence occurred naturally within a viral RNA genome (Shu & Guo, 2003). In this study, Shu & Guo found a similar sequence to the consensus sequence that had been frequently arrived at from previous ATP aptamer binding studies, was found to occur in Phi29-encoded 120 base pRNA (packaging RNA). pRNA is a non-structural RNA molecule, which naturally helps form the DNA filled capsid of the virus [REFS]. The pRNA forms a secondary structure that holds the ATP within in it until it is required by the DNA packaging motor (Parris *et al.*, 1988). The close proximity of the ATP allows the packaging of the viral DNA into the capsid to occur at an accelerated rate (Morita *et al.*, 1993).

1.8.2 Thrombin

Thrombin is a 36,000 Da serine protease that is found in mammalian blood and is the final active product of the blood clotting cascade (Wolberg, 2007). Thrombin is derived from prothrombin, a 72,000 Da protein, from which the active protein is cleaved by Factor Xa to produce two thrombin molecules for every single prothrombin. Thrombin then cleaves fibrinogen into fibrin which is then hydrolysed into a mesh of fibres in conjunction with platelets to provide a scaffold for the formation of blood clots (Lenting *et al.*1998).

The subject of one of the first examples of *in vitro* selection of aptamers was to human thrombin (Bock *et al.*, 1992). Following the initial isolation, the thrombin binding aptamer has been thoroughly characterised by several studies adding to the understanding of aptamer to target interactions and binding (Wang *et al.*, 1993, Macaya *et al.*, 1993, Paborsky *et al.*, 1993), this has been put to good use in the form of starting points and reference interactions for the development of further aptamer based technologies utilising aptamer to target interactions (Rye & Nustad, 2001, Hu *et al.*, 2009, Xie *et al.*, 2009). The outcome of this wealth of knowledge is that any potential biosensor, or biosensing technique, tends to be tested using ssDNA thrombin aptamer binding its target.

The thrombin aptamer consensus sequence GGTTGGTGTGGTTGG (**Figure 16**) folds into a intramolecular quadraduplex with the guanine duplex forming around a metal ion, such as K^+ which serves to stabilise the secondary structure (Kumar & Maiti, 2004, Wilcox *et al.*, 2008)).

Figure 16 3D image of a complex of Human Alpha-thrombin with the thrombin binding aptamer GGTTGGTGTGGTTGG based on X-ray diffraction data. A predicated computer model of complex between thrombin and the thrombin binding aptamer produced from data from a crystallographic and NMR solution structure (Padmanabhan et al., 1993 and Padmanabhan et al. 1996)with the thrombin depicted as an amino acid rainbow and TBA rendered in beige.

1.8.3 Whole Cell

One of the most successful example of whole cell-SELEX is the study carried out by Shangguan *et al. (Shangguan et al., 2008a)* targeting protein biomarkers with the aim of identifying disease specific protein expression. The DNA aptamers were selected against a cell line known to carry the disease T-cell acute lymphoblastic leukaemia (T-ALL), and from the resulting library one aptamer in particular (scg8) was found to have a high affinity for most of the T-ALL cells along with some specificity for acute myeloid leukaemia (AML) and B-cell acute lymphoblastic leukaemia (BALL) cells.

1.9 Biosensor design

The biosensor concept as mentioned above relies on a high affinity, highly specific molecular recognition element. Aptamers may provide a useful solution in the quest to determine the best possible resolution to this problem. Once the potential of aptamers as the MRE in a biosensor can be realised their place in combination, substitution, or, eventually as dominant alternative can be attained due to the ease of aptamer isolation demonstrated herein.

1.9.1 Modified ELISA system

An existing method that utilises antibodies as a molecular recognition element within a biosensor is ELISA. This technique requires the use of an antibody as a recognition molecule that will specifically bind an antigen, the detection of the binding event is then probed using a secondary antibody that has been raised to the primary (recognition) antibody. Attached to the secondary antibody is a reporter molecule, typically a fluorophore that can be detected at very low concentrations (nM to pM) allowing detection of an antigen at concentrations much below those required for bulk analysis such as absorbance spectroscopy (GoldFarb, 1951)

With the aim of displaying the benefits of aptamer technology as a possible alternative to antibodies, the use of aptamers within a modified ELISA sandwich has not yet been fully investigated. Although some published work (Tennico *et al.,* 2010) does detail the use of aptamers as either the primary or the secondary portion of the ELISA sandwich (Ferreira *et al.* 2008), there is, as yet, no report of a complete ELONA (Enzyme-Linked Oligonucleotide Assay) sandwich functioning.

1.9.2 The Generic Biosensor Concept

The proposed use of aptamers within a generic biosensor is as follows; the concept is based on the ligand-enabled release of a motor protein and the subsequent detection of the motor activity. Figure 17 is a schematic diagram of the concept, which utilises polyclonal antibodies as the MRE and a series of enzymes to initiate, amplify and enable a signal. The antibody ELISA sandwich will form an around any analyte to which antibodies can be raised, e.g. bacteria, spores, viruses, and proteins.

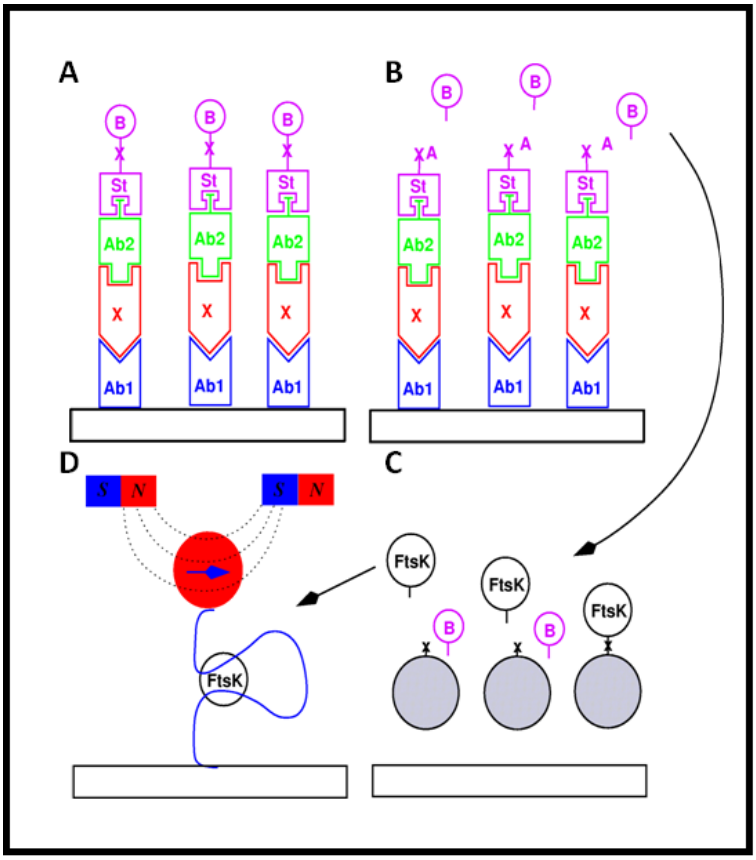


Figure 17 The Generic Biosenor concept. A. Ab1 and Ab2 form an ELISA sandwich around a target molecule. B. Ab 2 carries a streptavidin conjugation, to which a biotinylated protease B can be attached. Protease B can then be released by protease A. C. This protease will subsequently release a motor protein that has been bound to a surface by a peptide linker. D. This motor can then bind, then translocate a DNA strand that has been attached to a paramagnetic whose movement is then tracked.

The biosensor uses the release of the molecular motor from EcoR124I bound to create signal by translocation of a DNA strand that is attached to a magnetic bead whose movement can be tracked by either a magnetic tweezers system or by a Hall effect sensor. Immobilisation of this on a GST column has demonstrated the detection of glutathione through motor release (the fusion protein will translocate DNA). Unpublished results have shown motor release through thrombin cleavage on the column can give an amplification of greater than 1000 motors released by each thrombin molecule through cleavage.

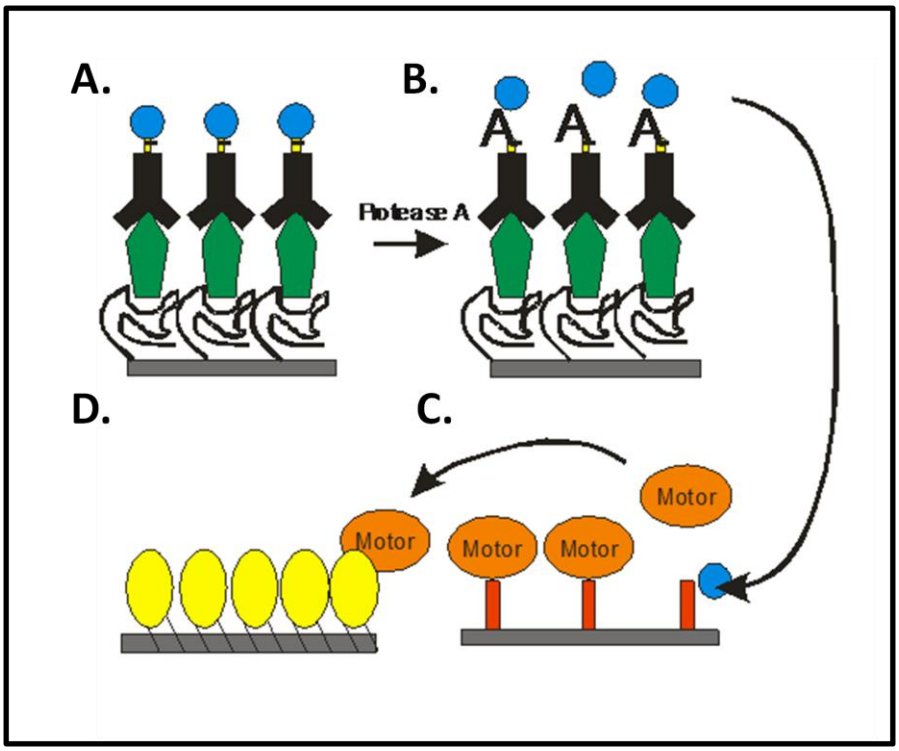


Figure 18 The proposed aptamer:antibody modified ELISA. *A.* Ligand1 and Ligand2 form an ELISA sandwich around a target molecule. B. Ligand 2 can carry a streptavidin conjugation, to which a biotinylated protease B can be attached. Protease B can then be released by protease A. C. This protease will subsequently release a motor protein that has been bound to a surface by a peptide linker. D. This motor can then bind then translocates a DNA strand that has been attached to a paramagnetic whose movement is then tracked as in the antibody ELISA version.

From the original model of a generic biosensor (Figure 17) Ligand 1 can be replaced by a ssDNA or RNA aptamer. As could Ligand 2, although Ligand 2 is not an absolute requirement for the system to work as the aptamer can carry the reporting ability (Figure 18). One possible use is to develop aptamers to toxin-receptors. DNA translocation will pull the magnetic bead past a sensor. The sensor could switch a silicon-based device. This allows an interface between the 'biological' world and the 'silicon' world, and it is the purpose of this thesis to demonstrate the ease that aptamers can be isolated and that aptamers be substituted for antibodies and can be the gold standard MRE of any biosensor.

2 Materials and Methods

2.1 Standard solutions

2.1.1 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-Gal)

20 mg X–Gal was dissolved in 1 mL dimethylformamide and stored at 4^{o}C until use.

2.1.2 Adenosine Trio Phosphate (ATP) Stock

0.05 g ATP, was dissolved in 1 mL 100 mM Tris.HCl pH 9 and stored at -80^{o}C until use.

2.1.3 Ampicillin stock

Ampicillin, sodium salt, dissolved in AnalR water to a concentration of 100 mg mL^{-1}, and filtered with a 0.2 µm filter. This stock was aliquoted, stored at -20^{o}C and used at a final concentration of 100 µg mL^{-1} when required.

2.1.4 Ammonium acetate 7.5M

57.81 g Ammonium acetate was dissolved in 100 mL and stored at room temperature.

2.1.5 Chloramphenicol

A stock solution of 20 mg/mL chloramphenicol was made by dissolving 20 mg of chloramphanicol in 1 ml of ethanol and later diluted to a working concentration of 50 µg/µL prior to use.

2.1.6 Coomassie Blue stain 1L

250 mg of Brilliant Blue R250 was dissolved in 500 mL Methanol, 100 mL Acetic Acid and 400 mL H_2O and stored at room temperature.

2.1.7 Destain 1L

100 mL of 100% Methanol was combined with 100 mL of Glacial Acetic Acid and 800 mL of H_2O and stored at room temperature.

2.1.8 500 mM EDTA

186.12 g di-potassium salt dehydrate (Sigma) was dissolved in 800 mL of AnalR water and then adjusted to pH 8. The solution was made up to 1 L with AnalR water and then autoclaved.

2.1.9 100mM HEPES

2.38 g of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), was dissolved in 100 mL of AnalR dH_20 and the pH adjusted to pH 7.5

2.1.10 HRV143C Cleavage buffer

2.42 g of Tris, 8.76 g of NaCl 0.154 g of DTT, and, 0.124 g of EDTA were dissolved in 1 L of AnalR dH_20 and the pH adjusted to pH 7.0.

2.1.11 1 M Isopropyl-β-D-thio-galactopyranoside (IPTG) Stock

1M IPTG stock solution was made by dissolving 2.38 g of IPTG in 10 mL of AnalaR water, followed by sterile filtering with a 0.2 µm filter. This stock was aliquoted into 1 mL and stored at -20^{o}C

2.1.12 10× Phosphate Buffered Saline (PBS)

80 g NaCl, 2 g of KCl, 11.5 g of Na_2HPO_4, and 2 g of KH_2PO_4 were dissolved into 100 mL of AnalR water and then autoclaved.

2.1.13 10 M Sodium Hydroxide

40 g sodium hydroxide (NaOH) (Sigma) was dissolved in 80 mL of AnalaR water. The solution was then made up to the final volume of 100 mL with AnalR water.

2.1.14 3× SDS Loading Buffer

30 % w/v Glycerol, 15 % w/v β-Mercaptol Ethanol (Sigma-Aldirch UK), and 9% w/v Sodium Dodecyl Sulphate, were dissolved in 1 mL 180 mM Tris pH 6.8 and stored at room temperature.

2.1.15 10× SDS–PAGE Running buffer

75.75 g of Tris(hydroxymethyl)aminomethane (Tris), 360.0 g of Glycine, and, 25 g of Sodium Dodecyl Sulphate (SDS) were dissolved in 2.5 L of dH_20.

2.1.16 Sodium Tris EDTA (STE)

5.84 g NaCl, 1.21 g Tris Base and 0.29 g EDTA were dissolved into 800 mL AnalR water, then pH adjusted to 8.0 before being made up to 1 L. The solution was then filter sterilised using a 0.2 µm filter, autoclaved and stored at room temperature.

2.1.17 50× Tris Acetate EDTA (TAE)

242 g of Tris base, 57.1 mL of glacial acetic acid and 18.6 g of EDTA were dissolved together and made up to 1 L with AnalR water. The solution was then filter sterilised using a 0.2 µm filter and stored at room temperature.

2.1.18 Thrombin Storage Buffer

0.11 g Sodium Chloride, 0.1 g Sodium Citrate were dissolved in 5 mL of AnalR dH_2O and adjusted to pH 6.5, to this Glycerol was added to 50% v/v and filter sterilised before storage at room temperature.

2.1.19 1 M Tris.HCl

1M Tris.HCl solution was made by dissolving 121.14 g Tris (hydroxymethyl)-aminomethane.HCl (Sigma) in 800 mL AnalaR water, then the pH was adjusted using concentrated Hydrochloric acid. The solution was then made up to 1 L with dH_2O water and sterilised with a 0.2 µm filter and then autoclaved.

2.2 Poly Acrylamide Gel Electrophoresis (PAGE)

2.2.1 10% Sodium Dodecyl Sulphate (SDS) Poly-Acrylamide Gel Electrophoresis

2.2.2 Preparation of Agarose gels

0.74 g agarose was added to 1.8 mL 50x TAE and made up to 90 mL with AnalaR water. The mixture was then boiled using a microwave and allowed to cool to 65°C in an appropriate water bath. Ethidium bromide was added to a final concentration of 0.5 µg mL^{-1} before pouring into the plate of a gel electrophoresis tank, with comb(s) inserted, and allowing the gel to set at room temperature. A running buffer of 1x TAE and 0.5 µg mL^{-1} ethidium bromide was poured into the gel tank ensuring the gel and wells were covered.

2.2.3 Agarose gel Electrophoresis

Gel electrophoresis was used to separate DNA fragments by running the samples at 50 V for 15 minutes then increasing to 100 V until full separation was observed. For fragment size comparison Bioline Hyperladder I was run alongside the loaded test samples.

Figure 19 **Diagram of the two DNA molecular weight markers Hyperladders I and V (Bioline)**

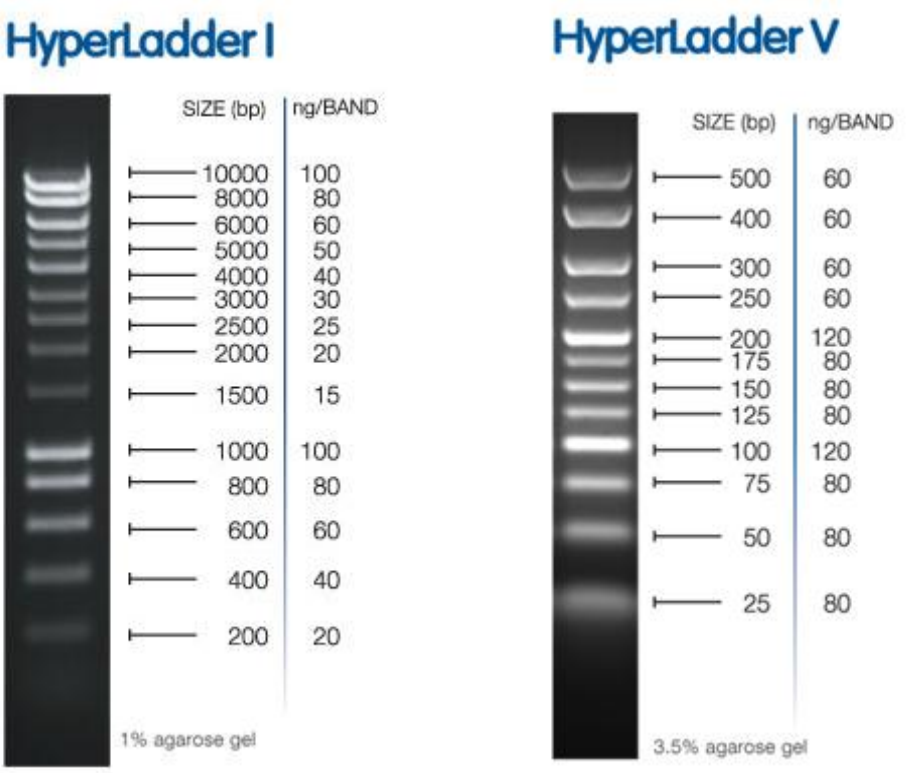


Table 1 Solutions required for the construction of 10% SDS PAGE gels

Solution A		Solution B		Solution C	
Acrylamide	30.4 g	**S.D.S**	0.4 g	**S.D.S.**	2 g
Bisacrylamide	0.8 g	**Tris.HCl pH 8.8**	18.2 g	**Tris.HCl pH 6.8**	30 g
dH_2O	100 mL	**dH_2O**	100 mL	**dH_2O**	100 mL

Table 2 Concentrations of solutions required for the construction of 10% SDS PAGE gels

Resolving Gel	Volume	Stacking Gel	Volume
Solution A	2.7 ml	**Solution A**	375 µL
Solution B	2.0 mL	**Solution C**	625 µL
H_2O	3.25 mL	**H_2O**	1.5 mL
Ammonium Persulphate	75 µL	**Ammonium Persulphate**	20 µL
TEMED	10 µL	**TEMED**	5 µL

The components listed in Table 1 and Table 2 were added together in the order listed and gels poured immediately. An interval of 30 minutes was allowed before electrophoresis In order to ensure that the gels had fully polymerised.

2.2.4 5% Native Gel

Chemical	Volume
Accugel (40%)	**2.5 mL**
50x TAE	**400 µL**
1M DTT	**60 µL**
H_20	**17.04 mL**
Ammonium Persulphate	**100 µL**
TEMED	**30 µL**

Constituents 1-4 were combined together before the sequential addition of ammonium persulphate and TEMED.The gel was poured immediately and left to set for 1 hour. The gel was then pre-run at 100 V for 1 hour at 4^{o}C before electrophoresis of samples.

2.3 Bacterial Strains

Strain:	*Genotype:*	Origin:
E. coli JM109	F', *traD36, proA^{+}B^{+}, lacI^{q}Δ(lacZ)M15/ Δ(lac-proAB), glnV44, e14^{-}, gyrA96, recA1, relA1, endA1, thi, hsdR17*	(Yanisch-Perron *et al.*, 1985)
E. coli JM109 (DE3)	*endA1, recA, gyrA96, thi-1, hsdR17(r_k-, m_k+), relA1, supE44, Δ(lac-proAB), [F', traD36, proAB, lacI^{q}ZΔM15], λ(DE3)*	(Yanisch-Perron et al., 1985)
E. coli BL21	*F^{-}, ompT, hsdS$_B$(r_B-, m_B-), gal*	(Weiner & Costa, 1994)
E. coli BL21 (DE3)	*F^{-}, ompT, hsdS$_B$(r_B-, m_B-),dcm,*	(Studier & Moffatt, 1986)

	gal, λ(DE3)	(Weiner *et al.*, 1994)
E. coli BL21 (DE3) pLysS	*F⁻, ompT, hsdS$_B$(r_B-, m_B-),dcm, gal, λ(DE3), pLysS (Cm^r)*	(Derman *et al.*, 1993)
E. coli DH5α	*Φ80lacZΔM15, recA1, endA1, gyrAB, thi-1, hsdR17(r_k-, m_k+), supE44, relA1, deoR, Δ(lacZYA-argF) U169, phoA*	(Woodcock *et al.*, 1989)

2.4 Plasmids

Plasmid:	*Function:*	*Reference:*
pGEX-2T	Ampr, GST-tag, thrombin cleavage site	(Smith & Johnson, 1988)
pGEX-6P	Ampr, GST-tag, PreScission Protease cleavage site	(Smith & Johnson, 1988)
pCFD30	Ampr, based on pUC19, contains a single EcoR124I site	(van Noort *et al.*, 2004)
pJS4M3	pJS4M based plasmid containing two tandemly arranged T7 gene 10 promoters	(Zinkevich *et al.*, 1997)
pGEM-T-Easy	Ampr, pGEM based plasmid containing T7 and SP6 RNA polymerase promoters. Linearisedwith 3' T overhangs.	(Kobs. 1997)
pGEX-2T-R	Ampr, GST-tag, thrombin cleavage site, containing HsdR gene.	Unpublished Plasmid
pGEX-6P-R	Ampr, GST-tag, PreScission Protease cleavage site, containing HsdR gene.	Unpublished Plasmid
pGEX-2T-PP	Ampr, GST-tag, thrombin cleavage site, containing gene for Human Rhinovirus 14 3C.	Unpublished Plasmid

2.5 Culture media

2.5.1 Luria Broth (LB) Media

Bactotryptone (10 g), Bactoyeast extract (5 g) and sodium chloride (5 g) dissolved in AnalaR water; adjust pH to 7.2 and made up to 1 L in AnalR water and autoclaved immediately.

2.5.2 LB Agar

Bactotryptone (10 g), Bactoyeast extract (5 g), sodium chloride (5 g) and bactoagar (15 g) were dissolved in AnalaR water; pH adjusted to 7.2 and made up to 1 L in AnalR water and autoclaved immediately.

2.5.3 2× TY Broth

Bactotryptone (16 g), Bactoyeast extract (5 g) and sodium chloride (5 g) dissolved in AnalR water; pH adjusted to 7.2 and made up to 1 L in AnalaR water and autoclaved immediately.

2.6 Microbial techniques

2.6.1 Liquid bacterial cultures

Liquid cultures of *E. coli* were grown in Luria Broth with the required antibiotic after inoculation with a single colony using an inoculating loop or sterile alternative. Cultures were grown at 37^{O}C with agitation at 180 rpm until the desired O.D.600 was achieved.

2.6.2 Preparation of competent bacterial cells for transformation

Cells of the required strain of *E. coli* were grown in an overnight culture in LB media at 37°C without antibiotic. 1 ml of the overnight culture was added to 100 ml pre-warmed LB medium in a 250 ml flask, and agitated at 180 rpm at 37°C until an O.D. of ~0.5 at a wavelength of 600 nm was reached. The culture was cooled on ice for 5 min, and transferred to a sterile, round-bottom centrifuge tube. The cells were harvested by centrifugation at 4000 x *g* for 5 minutes at 4°C. Cells were resuspended in cold 100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, buffer the suspension was kept on ice for an additional 90 minutes. The cells were collected by centrifugation at 4000 x *g* for 5 minutes at 4°C. The supernatant was discarded. the cells were resuspended carefully in 4 ml of ice-cold 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8 buffer. Aliquots of 100 µL were prepared in sterile microcentrifuge tubes and frozen in liquid nitrogen, or in a dry-ice-ethanol mixture. Competent cells were stored at –70°C and defrosted for use when required.

2.6.3 Transformation of Competent cells

10 ng of Plasmid DNA of the required concentration was added to an aliquot of 100 µL of competent cells and incubated at 0°C for 20 minutes. The cells were then “heat shocked” for

90 seconds at 42°C and 500 µL of LB broth was added and the cells were transferred directly to an incubator at 37°C for 1 hour with agitation at 180 rpm. The cells were then centrifuged for 15 seconds at 10,000 *x g* and resuspended in 200 µL of LB broth before spreading on an LB agar plate containing the required antibiotic. The plate was then incubated overnight at 37°C.

2.7 DNA Techniquess

2.7.1 Cloning and expression of aptamer sequences

The *Taq*. polymerase amplified the library but *Taq.* leaves a 3' adenosine overhang which faciliatated the ligation of the sequences into the pGEM-T-Easy plasmid which has a 5' thymine overhang. The ligation was performed overnight at a temperature of 4^{o}C to ensure complete ligation of the products. Following ligation the products were transformed into E.Coli [JM109] and analysed using the blue-white test (Maniatis *et al.*, 1982). The blue-white test operates by the insertion of a gene into the multiple cloning site of a vector that has been cleaved by restriction enzymes within the LacZ gene. This disruption prevents the production of functional β-galactosidase. By incubation in the presence of 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-gal), the cells lacking the β-galactosidase cannot metabolise the X-gal to form 5-bromo-4-chloro-indoxyl. When metabolised the X-Gal is oxidized 5,5'-dibromo-4,4'-dichloro-indigo, which gives the colonies a blue pigment. A blue colony indicates the hydrolysed oxidized 5,5'-dibromo-4,4'-dichloro-indigo and therefore the gene and the vector is intact. White colonies indicate insertion of foreign DNA and loss of the cells' ability to hydrolyse the marker. White colonies displaying the insertiong were then placed in a nutrient broth and incubated at 37^{o}C overnight before the plasmid DNA was isolated using QIAgen miniprep plasmid isolation kit. The recovered DNA was then sent for sequencing.

2.8 Silver staining

2.8.1 Staining procedure

The gel is fixed by incubation with gentle agitation in 100 mL of fixing solution, 50 % methanol, 10 % glacial acetic acid, for 1 hour. The the fixing solution is refreshed with a further 100 mL change of the fixing solution (gel may be left in the second change fixing solution overnight). After removal of the solution the gel was rinsed for 30 minutes in

100 mL of 5 % methanol, 7 % acetic acid with agitation. The solution is removed and is followed by an agitated wash for 30 minutes in 100 mL of 10 mM gluteraldehyde solution. The gel is washed thoroughly by 4 instances of a 15 minute incubation in AnalR dH_20 then followed by a 30 minute agitated incubation in 100 mL of 77 µM DTT solution. After washing the gel is stained by incubation in 100 mL of 1 mM Silver nitrate ($AgNO_3$) for 45 minutes then washed a further 3 times with freshly prepared developing solution (37 mM Formaldehyde, 3 mM sodium carbonate) for 1 minute. The gel was then agitated in fresh 100 mL of 100% developing solution for 4 to 15 minutes or until the bands reach desired intensity.

The developing reaction is stopped by the addition of 5 mL of 2.3 M citric acid to neutralise the developing solution and incubation for 15 minutes. The gel is washed again by 4 instances of 15 minute incubation in AnalR dH_20 then incubated with a 0.03% sodium carbonate solution for 15 minutes to prevent bleaching. The stained gel can then be stored at this stage in 20% methanol 5% glycerol 0.03% sodium carbonate in sealed plastic bag, or dried.

2.9 Selective Evolution of Ligands using EXponential Enrichment (SELEX)

100 µM Aptamer library of ssDNA was created by the dilution of 100 nmoles in 750 µL of nuclease free water and stored at -20^{O}C. The library sequence consisted of two known primer regions flanking an unknown 30 base pair random region:

GGC GGC GTA TTA TAN NNN NNN NNN NNN NNN NNN NNN NNN GCG CTA TAT

SELEX Forward Primer: CCG CCG CAT AAT AT

SELEX Reverse Primer: ATA TAG CGC

2.9.1 Glutathione Sepharose Spin Column Preparation

In a G-25 spin column (GE Healthcare®) a premixed suspension of 300 µL Glutathione Sepharose (GE Healthcare®) beads re-suspended added to the column and spun in a micro centrifuge at 1000 *x g* for 1 minute. 300 µL AnalR water was applied to the column and subsequently spun at 1000 *x g* for 1 minute and repeated three times to completely wash the column. The column is equilibrated by three sequential washes of 300 µL of the storage buffer required by the GST-fusion protein target each followed by a 1 minute spin at

1000 *x g*. 50 µL to 100 µL of the target protein is bound to the column media by incubation with together at ~21 ^{o}C for 10 minutes, any of the unbound remaining protein is removed by a spin in a micro centrifuge at 1000 *x g* for 1 minute. The column is washed with 300 µL of the specified buffer which is centrifuged at 1000 *x g* for 1 minute to removed excess buffer and repeated three times to ensure complete removal of unbound protein.

2.9.2 Screening

Following preparation of the column, 50 µL of aptamer library was introduced to the column and allowed to bind by incubation at ~21 ^{o}C for 10 minutes. Following incubation 300 µL of the required protein buffer are applied to the column with the removal of any unbound species removed with by centrifugation at 1000 *x g* for 1 minute. The washing step was then repeated three times to ensure a complete wash of the column. Elution of the bound species was achieved by the introduction of 100 µL 2 M $NaCl_2$ elution buffer, which was incubate for 10 minutes at ~21 ^{o}C. Following incubation the eluent is collected by centrifugation at 1000 *x g* for 2 minutes and desalted using GE healthcare G-25 desalting spin column.

2.9.3 Amplification

The Resulting DNA is amplified using 25 µL 2 x GoTaq Polymerase master mix, 1 µL SELEX Forward primer, 1 µL Reverse primers and incubated using the following protocol; Denaturation step of incubation at 94^{o}C for 3 minutes, then 94^{o}C for 1 minute, 60^{o}C for 30 seconds, and, 68^{o}C for 20 Seconds which is repeated 36 times. Then, a final extension of 68^{o}C for 1 minute 30 seconds followed by a 6^{o}C soak.

The screening and amplification cycles are repeated for 10-13 rounds, with each round analysed on a 15% native acrylamide gel, or non-denaturing HPLC using the Dionex 3000LC system.

2.10 Dot Blot analysis

Decreasing concentrations of protein to be probed were spotted onto nitrocellulose membrane in 1 µL dots and left to dry in air. The blots were then left to incubate overnight at 4^{o}C in 200 mL 1x TBST (5% BSA). After 16 hours the blots are then separated into strips according to the potential aptamer being tested and incubated for 2 hours in 10 µM of 3' biotinylated aptamer solution. The blots are then washed three times for 15 minutes in

200 mL 1x TBST (5% BSA), then in a 5 ml solution of the streptavidin-HRP conjugate, with the conjugate diluted 1:5000 in 1x TBST (5% BSA), for 2 hours at room temperature with agitation. The blots were then washed three times for 15 minutes in 200 mL 1x TBST (5% BSA) and then three times in 1x TBST for 5 minutes, following which a there was a final wash of 1 x PBS in which the blot was left until development.

The blots were then developed by combinination with a mixture of two solutions; Solution 1 : 100 µl Luminol, 44 µl pCoumeric Acid, 1 ml Tris HCl pH 8, 8.9 ml dH_2O, and Solution 2 : 6 µl H_2O_2, 1 ml Tris HCl pH 8, 9 ml dH_2O. After 1 minute the dot blots were then visualised using Fuji FLA3000 with a 0.85 mm aperture and an exposure time of 1 minute or longer if needed.

2.11 Surface Plasmon Resonance (SPR)

Standard Surface Plasmon Resonance experiments were carried out on the Biacore T100 (GE Healthcare) with buffers selected according the experiment undertaken. All buffers were made to 1 L and filtered and degassed before use.

Biotinylated aptamers were immobilised on a streptavidin coated Biacore SA chip in aptamer binding buffer (20 mM Tris pH 8, 10 mM $MgCl_2$) at 10 µL/minute flow following three 10 µL injections of 1M $NaCl_2$, 50mM NaOH to clean and prepare the surface. Sequential injections of 5 µM aptamer of increasing volume in binding buffer, from 1 µL to 15 µL aiming for an immobilisation level of 1000 arbitrary response units.

Kinetics of binding of the analyte was assayed by flowing samples of increasing concentration over the surface bound aptamers.

2.12 Purification of GST-PP-HsdR and GST-PP

10 ng of the plasmid pGEX-6P-R was transformed into competent *E. Coli* [BL21(DE3)] and incubated overnight at 37^{o}C. From this transformation single colonies were picked and grown in overnight cultures of 10 mL Luria Broth with 10 µg/mL ampicillin. After incubation 500 mL cultures of 2x TY with 10 µg/mL ampicillin were inoculated and incubated at 37^{o}C with 180 rpm agitation until a 600 nm optical density of 0.6-1.0 was reached. Once the optical density was reached the cells were induced with 1 mM IPTG and incubated overnight at 24^{o}C with 180rpm shaking. The following morning the cells were harvested by

centrifugation at 7000 *x g* for 12 minutes. The cell pellet was then resuspended in sonication buffer (1x PBS, 1mM DTT, 1mM Benzamidine, 0.1mM PMSF, 2 non-EDTA protease tablets.) and sonicated at a 40% amplitude for 3.3 seconds then 9.9 seconds recovery for a total of 15 minutes. The sonicate was then centrifuged at 27,000 *x g* for a total of 30 minutes, then the supernatant transferred to ultra-centrifuge tubes and centrifuged at 200,000 *x* g for 2 hours. The supernatant was loaded onto Glutathione Sepharose™ Fast Flow column (GE Healthcare) that was pre-equilibrated with PBS, 1 mM DTT and then buffer exchanged into buffer A (10 mM Tris.HCL pH8, 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT) and purified GST-tagged protein eluted with Tris buffer B (10 mM Tris.HCL pH8, 0.2 M NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM Glutathione) into 4 ml fractions. Any peaks were investigated using SDS-PAGE and any fractions found to contain protein of the correct size were pooled. Free Glutathione was removed by using 100,000 MW cut off spin columns (Centricon©). Depending on the application, the purified protein was used straight away or made to 50% glycerol for long term storage at 20^{o}C.

2.13 Ecor124I HsdM Subunit Isolation

10 ng of the plasmid pJS4M3 (Patel & Firman, 1992) was transformed into competent *E. Coli* [JM109 (DE3)] and left to incubate overnight at 37°C, from this 20 colonies were selected at random and incubated in 3 mL Luria broth at 37°C with agitation at 180 rpm over 8 hours. After incubation 1 mL samples were taken, these samples were then spun at 13000 *x g* for 1 minute to pellet the cells after which the cells were resuspended in 30 µL SDS loading buffer and boiled at 100 °C for 5 minutes. All 20 samples were then run on a 10% SDS PAGE gel and expression of the wild type HsdM subunit was estimated using a semi-quantitative ocular assay. The colony exhibiting the greatest expression level was selected for further growth in large scale culture. The cells were then grown for 16 hours at 37°C with shaking at 180 rpm, and then harvested by centrifugation at 3,500 *x g* for 30 minutes. The cell pellet was resuspended in sonication buffer (50 mM Tris.HCl pH 8.0, 20% glucose, 3 mM DTT, 5 mM EDTA, 0.1 mM PMSF, and 1 mM benzamidine) and sonicated with 3.3 s bursts of 40% amplitude and a recovery time of 9.9 s for a total time of 10 minutes. The cell lysate was then centrifuged at 27,000 *x g* for 30 minutes to clear the sonicate and followed by a 2 hour centrifugation step at 200,000 *x g* to clear the majority of cell debris from the lysate. The supernatant was then adjusted to 500 mM NaCl, 10 mg/mL protamine sulphate, and rocked

gently at 4°C for 30 minutes from which any DNA was pelleted by centrifugation at 27,000 *x g* for 30 minutes. The supernatant was then subjected to a 70% ammonium sulphate fractionation and centrifugation step of 27,000 *x g* for 30 minutes. The pellet was then resuspended in buffer A (50 mM Tris.HCl pH 8.0, 50 mM NaCl, and 1mM EDTA) and desalted using a pre-equilibrated HiTrap™ desalting column (GE Healthcare) in tandem (10 mL total volume) and eluted of a 100 mL gradient of 50 mM to 500 mM NaCl using buffer B (50 mM Tris.HCl pH 8.0, 1 M NaCl, and 1mM EDTA). Fractions found to contain the wild type HsdM subunit were then pooled and desalted into buffer C (50 mM Tris.HCl pH 8.0, 100 mM NaCl, and 1mM EDTA) using the previously described method above. After desalting the pooled fractions were loaded onto a HiTrap™ Heparin column (GE Healthcare) and the protein eluted using a 100 mL gradient from 100 mM to 1 M NaCl using buffer B. The fractions found to contain the HsdM subunit were pooled and made to a 50% glycerol solution and stored at -20°C until required

3 Analysis and/or characterisation of a known aptamer

3.1 Thrombin

The aim of this chapter is to demonstrate the binding of a known aptamer through widely available molecular laboratory techniques. Presented here is the application of two techniques; Fluoresence Resonance Energy Transfer (FRET), and Surface Plasmon Resonance (SPR), in the analysis of binding of a known aptamer (TBA), to its target (thrombin).

The thrombin binding aptamer, (TBA) first described by Bock *et al.,*(Bock *et al.*, 1992) is a well defined DNA aptamer and as further studies have been published the mechanism of folding and binding of the aptamer to thrombin has been thoroughly understood. The aptamer occupies the heparin binding site of thrombin mimicking the molecule's footprint on the active site and preventing binding of thrombin to fibrinogen with similar efficiency to the chemical(Bock *et al.*, 1992). Using this stable aptamer to target binding partnership, further aptamer binding validation methods can be developed with reference to the published characteristics and binding affinities.

3.2 Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two molecules in which excitation is transferred (Runnels & Scarlata 1995) from a donor molecule to an acceptor molecule without the emission of a photon (Figure 20). FRET can be an important technique for investigating a variety of biological processes which result in changes in the molecular proximity of two known components, for example the cleavage of a peptide with a fluorescent molecule and a quenching molecule in close proximity would result in an increase in fluorescence (Figure 21). FRET can be used as a contrast mechanism, that is, to probe the co-localization of proteins and other molecules. This facilitates a method of imaging and an insight into spatial resolution, which is far beyond the limits of conventional optical microscopy (Hell, 2009).

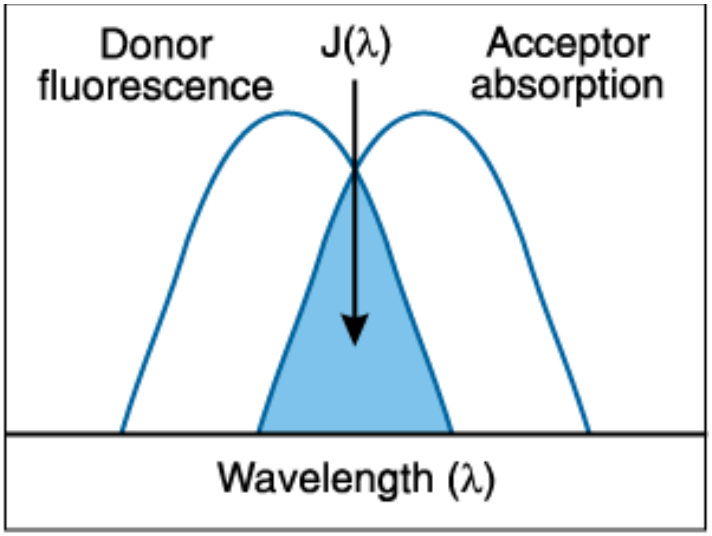


Figure 20 Diagram of electron transfer overlap in FRET. Schematic representation of the FRET spectral overlap, where J is the emission wavelength required for the absorption that results in the excitation of the acceptor between a quencher and emission FRET pair. Http://probes.invitrogen.com/handbook/boxes/0422.html

The ability to report whether two molecules are in close proximity can be used to detect the presence or absence of a cleavage enzyme, be it nucleic acid or peptide. The detection of cleavage can also be extended to an inhibitory agent preventing cleavage. In a method similar to the molecular beacon concept (Tyagi & Kramer, 1996) the cleavage assay will report the cleavage of a peptide via the change in signal achieved when cleavage occurs. Such an event can occur when thrombin cleaves its substrate peptide in imitation of the protease's role within the blood clotting cascade (Esmon, 1995). If a cleavage event does happen, the quencher and donor molecules are a sufficient distance apart for the quenching effect to cease and fluorescence will occur (Figure 21). Assuming efficient cleavage of the peptide by the protease an inhibitory effect can be observed and possibly calculated by a reduction in signal. Inefficient cleavage can be caused by inappropriate conditions such as incorrect buffer constitution, pH or temperature, or an inhibitory agent. In the case of the serine protease thrombin, its proteolytic activity is inhibited by a number of chemical compounds, heparin and warfarin notwithstanding; there are approximately 50

commercially available drugs available that reportedly inhibit thrombin's coagulant ability (Levy *et al.*, 2007). The common feature of the thrombin inhibitors is the occupation of the catalytic cleft that acts as the active site for the digestion of soluble fibrinogen to its constituent individual fibrils. The occupation can be achieved by a steric mimicry of the substrate fibrinogen and a greater affinity for the inhibitor preventing substitution. The Bock thrombin inhibitor aptamer uses this method to occupy the heparin binding site with a dissociation constant of ~ 60 nM and subsequently reduces coagulation accordingly (Bock et al., 1992).

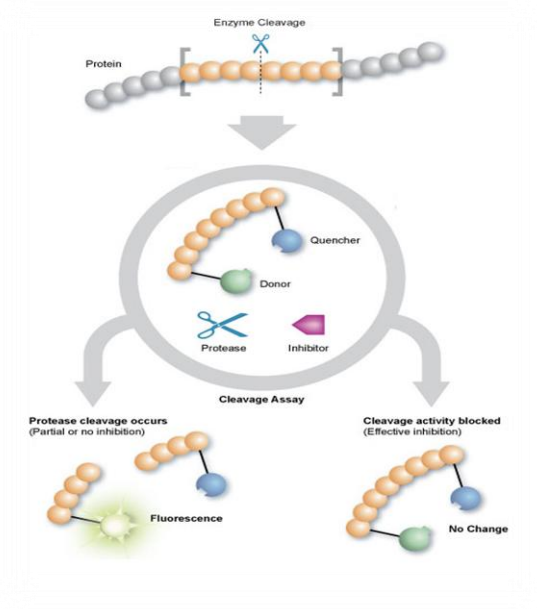


Figure 21 Cleavage Assay diagram, Diagram depicting the mechanism through which peptide cleavage can be converted to a FRET response. http://www.mimotopes.com/Uploads/Images/peptides-and-antibodies/Advanced-FRET-Principal-3a.jpg

With reference to the FRET detection system, the peptide carried a fluorescence reporting mechanism along with a peptide sequence containing the recognition and cleavage site for α-thrombin (GLVPRGSG)(Chang, 1985). Assuming the efficient cleavage of the substrate an increase of FRET can be measured by increasing fluorescent signal in a concentration dependent manner. Conversely a decrease in signal not attributable to inefficient cleavage can be assumed to be due to an inhibitory effect such as the presence of an aptamer inhibitor. It is this effect that may give an insight into the inhibitory effects of the Bock thrombin binding aptamer.

3.3 Surface Plasmon Resonance (SPR)

The technique of SPR relies upon a physical anomaly that occurs at the interface of light and particular metals e.g. Gold, silver or copper it creates an evanescent wave due to the creation of polaritons or "plasmons" resulting in a phenomenon called the plasmon effect. This field has a unique characteristic that it will deflect light by a discrete angle that is reliant on the nature of the surface it is emitted from. The deflection of a laser beam can be measured with a high level of accuracy giving rise to the ability of being able to detect mass applied to the reverse of the gold surface. The detection of mass can be so sensitive it is possible to determine and measure quantities that are within the nano- and picogram scale. Modern approaches also allow the tracking of molecular interactions making it possible to interpret and quantify results with the aim of deriving binding affinities and dissociation constants for the interaction being studied (BIACORE, 1998).

Surface Plasmon Resonance has a number of advantages that set the technique apart from other systems. A major benefit of the method is the ability to provide and to collect real-time kinetics data for association and dissociation reactions. As only one of the binding partners need be immobilised to the surface labelling of the other molecule/s is not necessary, labels and tags are known to interfere with binding, by both inhibiting and assisting binding (Liedberg *et al.*, 1995). So, the benefit of using at least one or two unlabelled factors will serve to remove some doubt as to the stringency of the recovered data concerning affinity.

SPR relies on the shape of molecules interacting with the surface. It can give an idea of mass because of the size of molecule or compound interfering with the plasmon effect but not discrete numbers regarding an accurate molecular weight or density. Alternatives to SPR that can provide reliable information regarding mass and density are Dual Polarisation Interfereometry (DPI) and using a Quartz Crystal Microbalance (QCM). A major disadvantage of the technique also lies in one of its strengths. As the technique requires the immobilisation of one of the analytes, the surface is required to have some properties that enable molecules to interact and to bind to it. As the gold surface and dextran matrix carry an electrostatic charge in low (3-4) pH's some molecules can interact and bind to the surface in a non-specific manner depending on the pH of the solution they are dissolved in. As a result the surface can interfere with binding reaction through this method and also through

steric hindrance. Steric hindrance can occur when the binding site of the immobilised component is too close to the surface preventing the other binding partners from access by sheer physical size.

It is through this method that growing number of aptamer:antigen interactions have been studied (Wang *et al.*, 2007), the thrombin binding aptamer for example has been studied in this method twice giving an estimated dissociation constant of Kd = 0.5 nM (Tang *et al.*, 2007a, Balamurugan *et al.*, 2006). Although this method for characterisation of aptamers is not novel, it is prudent to develop and understand a suite of techniques that endow the project with the capabilities to assay any potential aptamers that may need to be defined. By immobilising a potential aptamer on a gold surface through a biotin:streptavidin linkage and passing over a known concentration of the aptamers target molecule it is possible to arrive at putative parameters of interaction.

This chapter demonstrates the ability to assay aptamer to target binding within a regular molecular laboratory with standard equipment. It demonstrates the characterisation and evaluation of aptamers in an efficient and reliable manner. The efficiency of aptamer binding is assayed using surface plasmon resonance (SPR), Fluorescence Resonance Energy Transfer (FRET), and Electrophoretic Mobility Shift Assay (EMSA). Each method presented here is analysed and evaluated as an exhibition of useful methods for the analysis of aptamer:antigen interactions.

3.4 FRET based Cleavage assay

3.4.1 Custom Peptide Cleavage Assay

The first step in the creation of a biosensor is the ability to confidently assay the components of a reaction that are pertinent to the user. In order to determine the usefulness of a technique a known and well characterised system is preferred. As mentioned above, the inhibitory effects of the thrombin binding aptamer are well reported (Bock et al., 1992), and the aim of these assay was to take advantage of this foundation of evidence and demonstrate aptamer binding through the inhibition of cleavage. After the construction of a calibration curve to determine the kinetics and reliability of a thrombin catalysed cleavage of the custom peptide, a titration of TBA can indicate the inhibitory effect of an aptamer on thrombin cleavage and be used to demonstrate binding.

In order to assay thrombin activity a FRET based assay was developed based on the cleavage of the peptide through the creation of a calibration curve using custom peptide received from mimotopes (H-(161)GLVPRGSG(160)-NH2) and thrombin protease. Concentration required for each assay determined with reference to the literature a concentration of 4 µM per reaction (Esmon, 1995).

Each 100 µl (total volume) reaction took place in one well of a 96-well plate with fluorescence measured on Biotek FL 600 Fluorescence and Absorbance Reader.Each reaction was carried out in 1 x PBS and concentration of the peptide required for each assay determined with reference to the literature a concentration of 4 µM per reaction. The samples were then incubated at 22°C for 2 hours and a fluorescence reading taken every 15 minutes.

A series of thrombin dilutions from 100 U/mL to 0.001 U/ mL (Table 4) were used and their activity monitored by measuring the final fluorescence output at 570 nm as presented in Table 3 and Figure 22.

Table 3 Thrombin Cleavage Calibration Curve construction. Table showing the results of the cleavage of the FRET substrate. Each well contained a 100 µL reaction consisting of: custom peptide 4 µM, 1x PBS, and α-thrombin at varying concentrations

	Thrombin concentration (U/ml)					
Time (minutes)	0.001	0.01	0.1	1	10	100
0	0	0	0	0	0	0
15	0	0	12	95	455	421
30	3	0	0	153	449	415
45	0	0	0	229	443	391
60	6	0	3	272	433	397
120	0	0	18	388	409	369

The data shown in Table 3 and graphically represented in Figure 22 highlight the shallower nature of the initial rate curve at 1 U/mL when viewed in contrast to the higher and lower concentrations of thrombin an inhibition of the initial rate may be observable. It is with this in mind that the concentration to take forward as a suitable assay for inhibition would be

1 U/mL any inhibitory effects by an aptamer would be detectable via a deviation from this known curve.

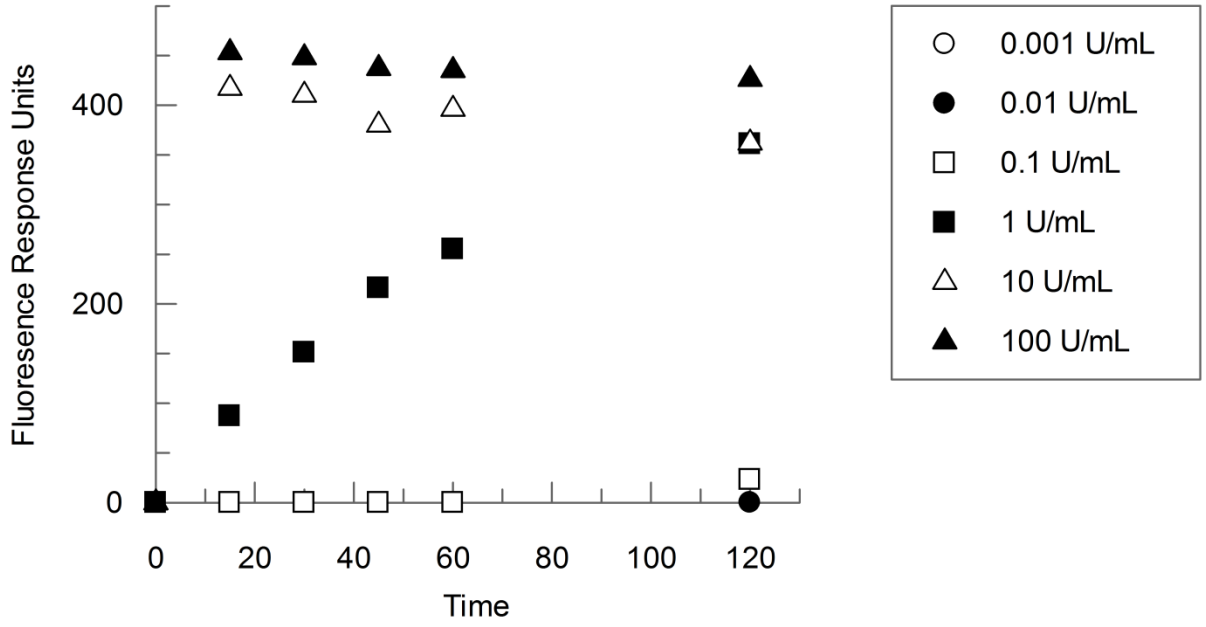


Figure 22 Thrombin Cleavage Calibration Curve of fluorescence against time. A graphical representation of the results of the cleavage of the FRET substrate (Table 3). Each well contained a 100 µL reaction consisting of: custom peptide 4 µM, 1x PBS, and α-thrombin at varying concentrations.

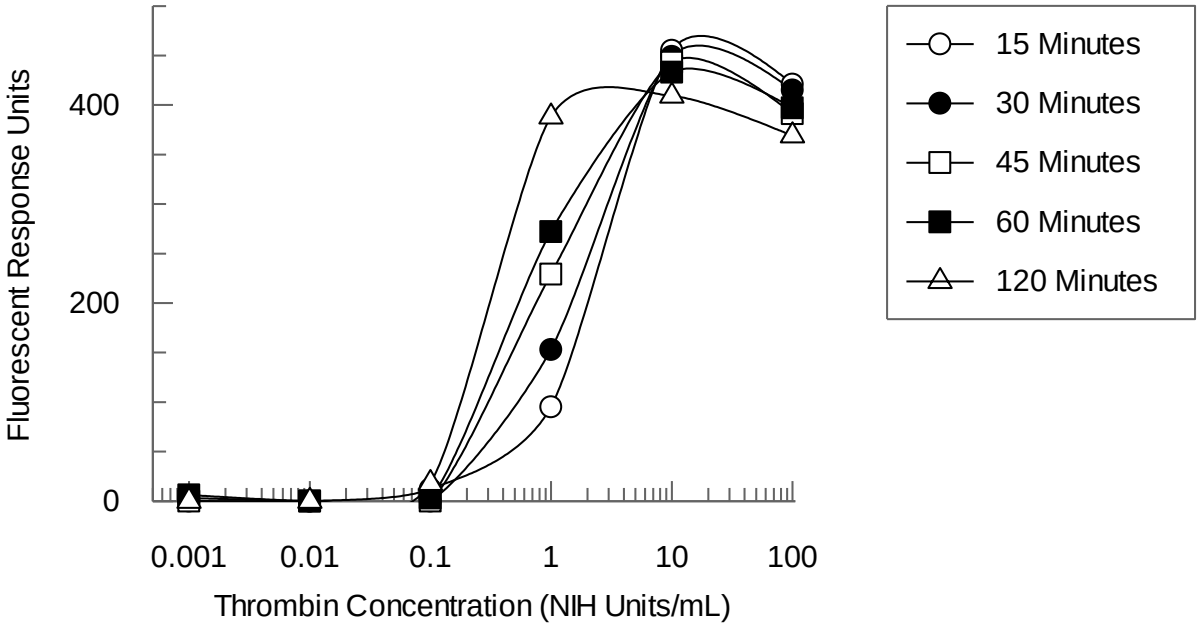


Figure 23 Thrombin Cleavage Calibration Curve concentration against RU. A graphical representation of the results of the cleavage of the FRET substrate. The data here shows the reaction rate is concentration dependant and that the limiting factor for the reaction can either be time or concentration. Each well contained a 100 µL reaction consisting of: custom peptide 4 µM, 1x PBS, and α-thrombin at varying concentrations.

From the graph of the results of the calibration curve construction (Figure 22) it is apparent that using 1 NIH unit/mL is best suited to the equipment and allows a comparable figure for the determination of initial rate to be arrived at after a 15 minutes incubation period. From a rearrangement of the data (Figure 23) a conclusion about the concentration dependent nature of the reaction can be drawn. The dissociation constant of the thrombin peptide interaction can be determined from this graph to be ~1 U/mL which is the equivalent to 2.73 µM. This is the same order of magnitude for the affinity constant of thrombin to its natural substrate fibrinogen which is 1.3 ± 0.3 µM (Mathur *et al.*, 1993) this corroborates the finding that this method can be assumed to provide reliable data relating to initial rate calculations.

3.4.2 Inhibition Assay

Following the successful creation of a calibration curve and a determination of desirable considerations for the assay of thrombin cleavage of the peptide, an inhibition assay was sought that would display aptamer binding through the inhibition of thrombin cleavage by three known aptamers (Bock et al., 1992). Using the pre-defined conditions, which remain constant throughout, titration of different concentrations of aptamers and the degree to

which they inhibit, can be solved because of deviations in the amount of fluorescence observed. This does assume that pipetting errors and concentrations are minimal and are as stated and suitable.

Table 4 Table detailing the construction of the cleavage assay

Thrombin Concentration (U/mL)	**0**	**0.1**	**0.5**	**1**	**5**	**10**	**50**	**100**
Aptamer (5 μM)								
15 minutes								
v1	5	9	15	92	85	49	137	107
v3	64	0	6	0	134	9	119	61
R70E	70	55	31	52	92	95	85	177
2 Hours								
v1	113	10	143	363	171	153	449	372
v3	296	76	92	0	620	10	436	345
R70E	302	162	244	330	394	381	430	507
Repeat								
Thrombin Concentration (U/mL)	**0**	**0.1**	**0.5**	**1**	**5**	**10**	**50**	**100**
Aptamer (5 μM)								
15 minutes								
v1	6	40	3	15	64	89	156	134
v3	37	61	18	89	95	76	122	55
R70E	49	55	67	89	58	61	49	34
2 Hours								
v1	27	165	76	110	269	314	388	372
v3	208	269	95	336	614	339	394	287
R70E	259	311	293	436	296	339	269	208

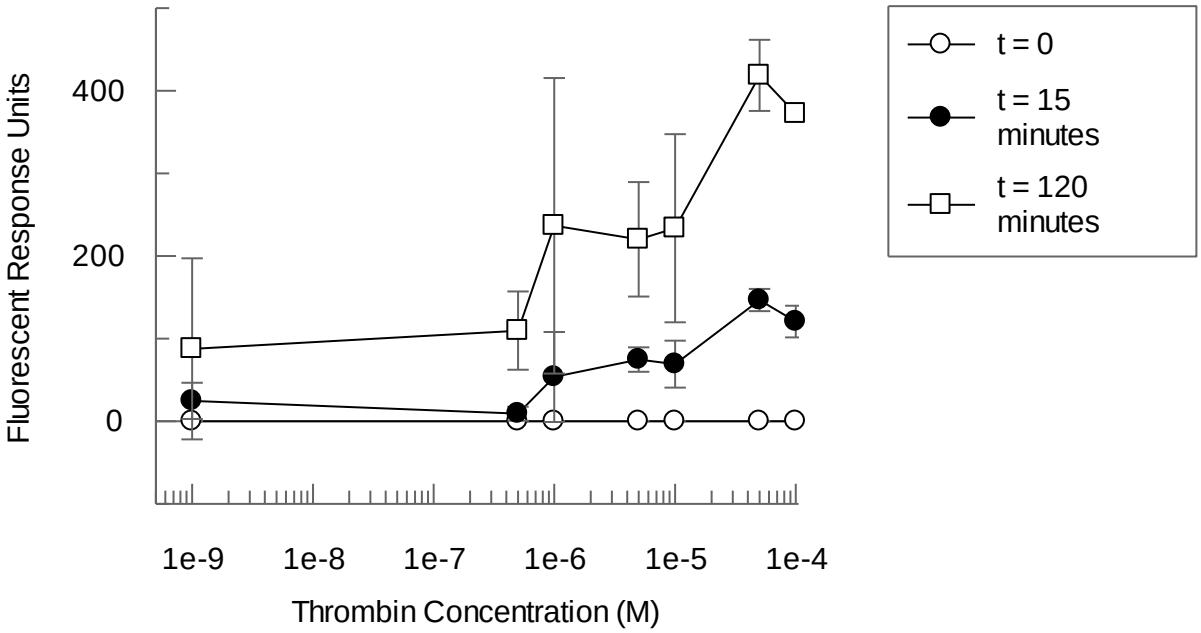


Figure 24 Inhibition of Thrombin-mediated Custom peptide cleavage Assay using anti-thrombin aptamer v1. Thrombin inhibition assay using anti-thrombin aptamer v1 as the antigen. Each reaction was assayed at 15 and 120 minutes. Fluorescent response observed at λ570 nm with 5 µM peptide, 5 µM aptamer, and increasing concentrations of thrombin in 1 x PBS at ~21°C.

The lack of reproducibility involved with the cleavage of thrombin is a worrying factor, although a slight downward trend can be observed this is against the literature. As the concentration of the aptamer increases so does the signal. Unfortunately this trend is replicated across the entire data set and can be found for the R70E hybrid aptamer also. The v3 aptamer (Figure 25) gives no noticeable trend in either direction except an anomalous result which should be instantly discredited as error.

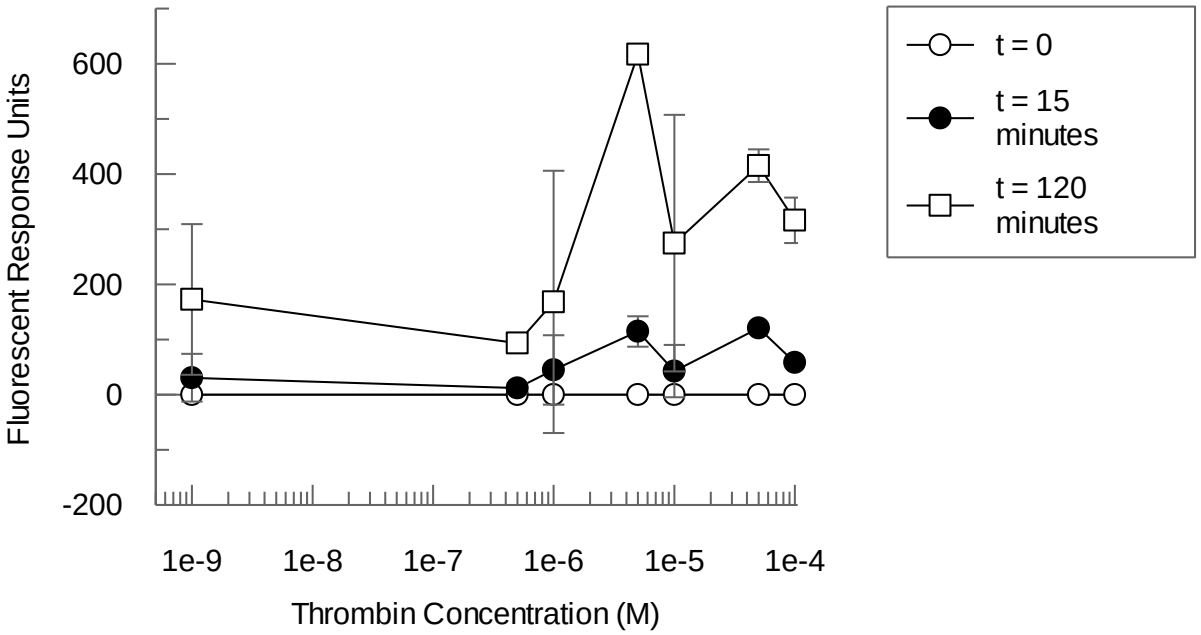


Figure 25 Inhibition of thrombin dependant custom peptide cleavage assay using anti-thrombin

aptamer v3. Thrombin inhibition assay using anti-thrombin aptamer v3 as the antigen. Each reaction was assayed at 15 and 120 minutes. Fluorescent response observed at λ570 nm with 5 µM peptide, 5 µM aptamer, and increasing concentrations of thrombin in 1 x PBS at ~21°C.

As discussed before, the trend across all three aptamer inhibition assays continues, this serves as further evidence of a sensitivity issue for this method. Inhibition is not demonstrable via this method as any deviation in the rate of cleavage of the custom peptide is evidentially outside the range of detection due to the sensitivity of the instrument employed for this task. It should also be noted that the peptide decays rapidly preventing the reproduction of results from one experiment to the next.

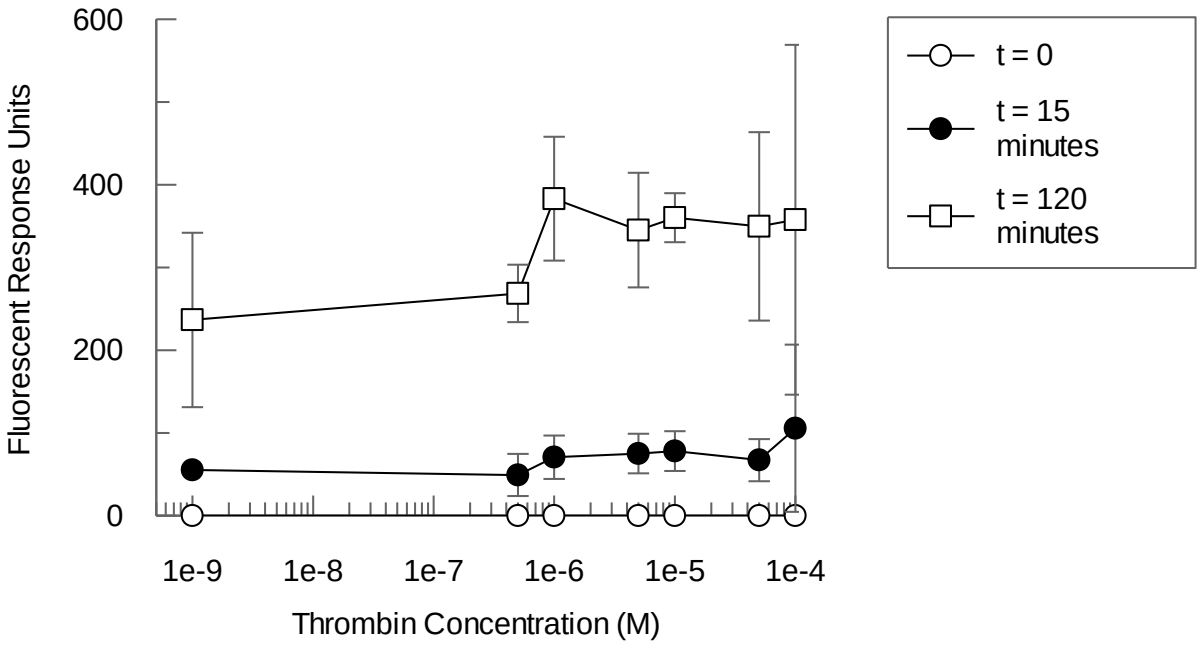


Figure 26 Inhibition of thrombin dependent custom peptide cleavage assay using anti-thrombin aptamer R70E. Fluorescent Response units observed after 15 and 120 minutes following the cleavage of the fluorescent peptide by thrombin according to reaction conditions detailed above. Thrombin Binding Aptamer R70E was present throughout at a concentration of 5 µM.

The results do not show any noticeable trends which could be the result of a number of possible issues. The most obvious of these is experimental error; if the same amount of peptide is not being added each time it would make it extremely difficult to be able to draw

any useful comparisons. If, as is seen here, the experimental error exceeds the all other variation it is impossible to draw any conclusions from the results.

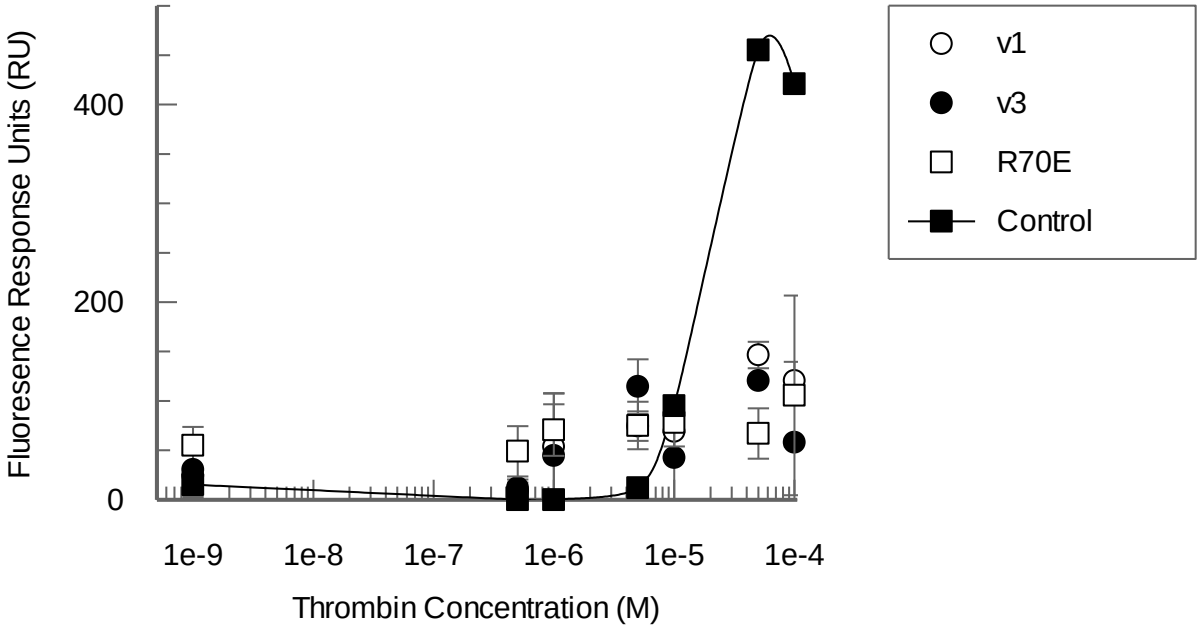


Figure 27 Inhibition of thrombin cleavage after 15 minutes. Fluorescent response observed after fifteen minutes at λ570 nm with 5 µM peptide, 5 µM aptamer, and increasing concentrations of thrombin in 1 x PBS at ~21°C.

After 15 minutes all three of the thrombin aptamers display some inhibition of the target with R70E producing the greatest amount of inhibition across all the concentrations (Figure 27). However, after 120 minutes the inhibition is less profound. This reduction in inhibition could be due to binding of the aptamer and target in equilibrium not being permanent and because of this the thrombin only need cleave the substrate once for a signal change. Alternatively, it may also be attributable to the degradation of the fluorescent peptide substrate as later work indicates some thermal instability which renders the peptide void (data not shown).

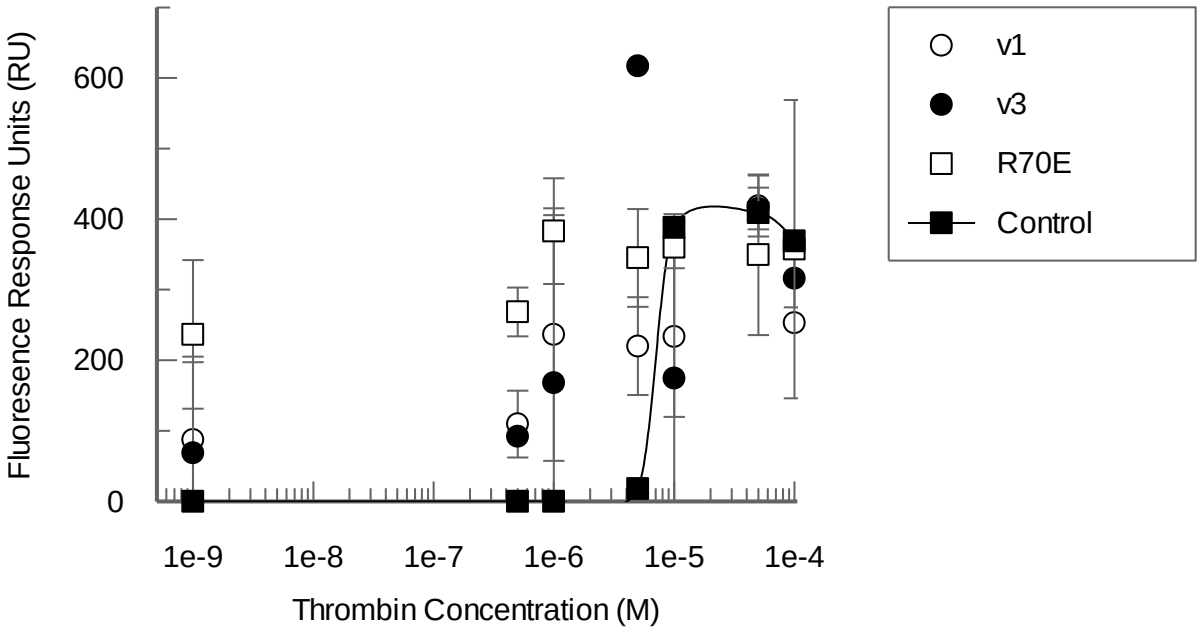


Figure 28 Inhibition of thrombin cleavage after 120 minutes. Fluorescent response observed after fifteen minutes at λ570 nm with 5 µM peptide, 5 µM aptamer, and increasing concentrations of thrombin in 1 x PBS at ~21°C.

This instability furthers the suggestion that peptide cleavage assay is unsuitable as a useful aptamer binding study. This instability will limit the conditions under which the assay is performed and negate one of the benefits of using aptamers, the point that aptamers will bind in a variety of conditions. This, coupled with the fact that the inhibition assay as presented here relies on the inhibition of proteolytic cleavage, would restrict the potential aptamer targets for derivation to proteases and other enzymatic targets that produce a known molecular event.

3.5 Surface Plasmon Resonance (SPR)

Three aptamers were selected from the literature (Bock et al., 1992) to bind thrombin at a range of affinities, thrombin binding aptamer v1, v3 and aptamer hybrid R70E were chosen to have relatively strong, medium and weaker affinity to thrombin respectively (Figure 29). Through the comparison of the interactions the results may give an insight into the nature of the interactions as it is possible to determine dissociation constants, binding affinities, and gain thermodynamic insight amongst others using this method.

The thrombin binding aptamer v1, being one of the first aptamers studied, is well defined in its adapted secondary three dimensional structure and the interactions of aptamer and antigen almost fully understood. The aptamer is understood to occupy the heparin binding

site of thrombin mimicking the drug's footprint on the active site (ref), preventing proteolysis with a comparable affinity to that of heparin. In order to further characterise the interaction of the thrombin binding aptamer to thrombin, Surface Plasmon Resonance (SPR) studies were performed. These studies aim to corroborate the data and conclusions drawn in the scientific paper from Tang *et al*. (Tang et al., 2007a) and determine our ability to detect and quantify thrombin within an unknown solution. If successful, the use of SPR as would provide a high-throughput and reliable method for the examination of candidate oligonucleotides that arise as the result of SELEX.

Aptamer sequences:

Thrombin Aptamer v1: GGTAGGTGTGGTTGGTGCAAC

Thrombin Aptamer v3: GGTAGGTTTGGTTGGTGCAACGC

Thrombin Aptamer R70EssP5: GGTAGGTAGGTATGGTGCAACGC

Figure 29 Consensus sequences used for Aptamer. Three aptamers were designed using the consensus sequence drawn from Bock (Bock et al., 1992). The affinity of the target to each individual ligand being relatively strong, medium, and weak.

3.5.1 Immobilisation of Aptamer candidates

Figure 30 depicts the successful surface attachment of aptamer v1 via a biotin streptavidin interaction. The reverse of the gold surface is supplied with streptavidin covalently bound to the dextran matrix via an amine linkage to functionalised carboxyl groups. The streptavidin coated SPR sensor chip and the 5' biotinylated aptamer v1 are incubated together with a low flow rate across the chip until the response units reach the required level. Injections of the aptamer solution fall at time points 410 s, 520 s, 570 s, 610 s with the desired level of response achieved after 675 s. After surface immobilisation the oligonucleotide can be assumed to be bound to the matrix with confidence, as the high affinity between streptavidin and biotin providing an almost irreversible and stable interaction that is in the order of $K_d=10^{-15}$ mol dm^{-3} (Green, 1975).

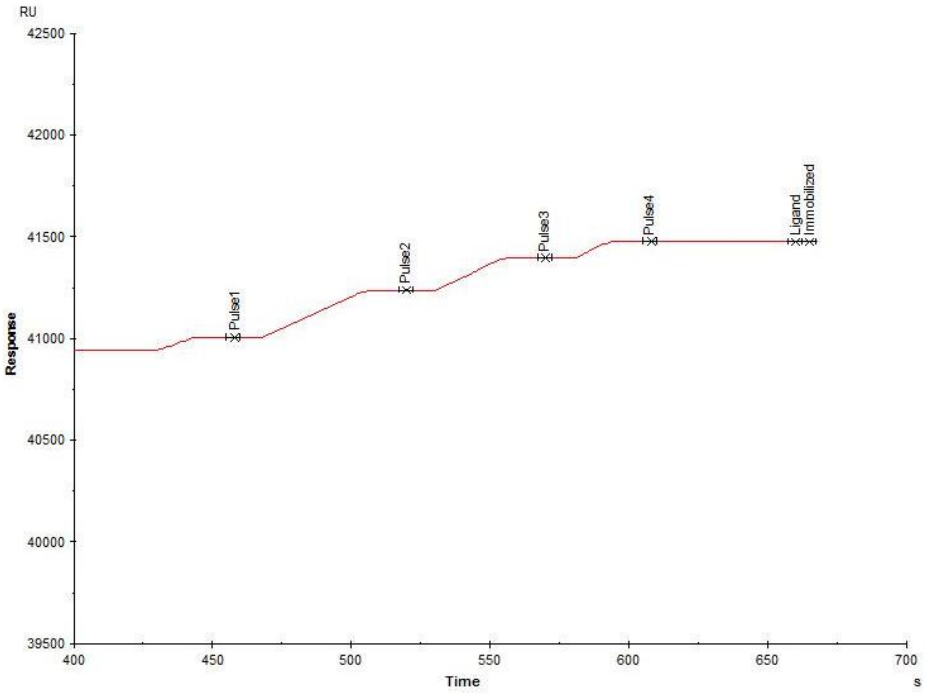


Figure 30 SPR sensorgram of biotinylated thrombin binding aptamer v1 immobilised to the streptavidin coated SPR chip. This figure shows the initial binding of the biotinylated aptamer to the streptavidin coated SPR chip (Biacore). 5 pulses of 1mM aptamer solution and wash steps of 50 mM NaOH to the required net change in response units of 1000 RU. After the fifth pulse and wash step the required response unit change was achieved indicating complete binding of the aptamer to the surface.

3.5.2 Probing the thrombin binding aptamer:thrombin interaction

Figure 31 is a sensorgram illustrating increasing concentrations of thrombin being passed over an SPR chip with TBA v1 aptamer bound. Thrombin concentration increases from 0 nM, 5 nM, 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM. The sensorgram is the response units of a channel containing the surface bound thrombin binding aptamer against a reference channel. Ruling out the possibility of non-specific binding interfering with the observed level of aptamer: thrombin interaction. Using only one channel for detection, thrombin is flowed over the chip-aptamer conjugate; with injection of increasing concentrations of thrombin starting at time point 0 s and halting after 20 s. Following the injection the dissociation rate can be deduced to be slow due indicating thrombin:aptamer interactions. When analysed, the apparent dissociation constant for the TBA v1 is approximately K_d=8.57 $^{-7}$ m which, is comparable with the initial findings for the thrombin binding aptamer of K_d= 0.25-2 $^{-7}$ m (Bock et al., 1992). Using the same method and

concentrations it was possible to define dissociation constants for TBA v3 and R70E which can be reported to be 1.5 µM and 0.2M respectively. These results corroborate the hypothesis that the candidate aptamers would be a strong, medium and weaker binding species.

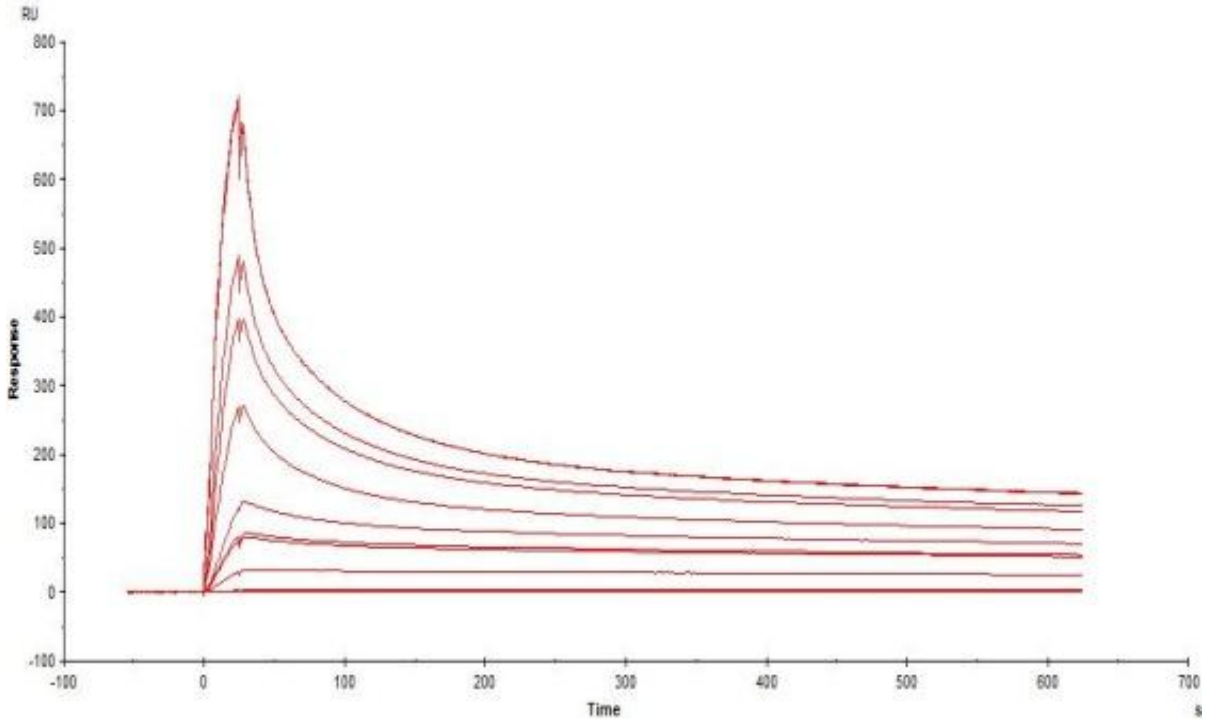


Figure 31 Increasing concentrations of thrombin passed over the SPR chip with the thrombin binding aptamer v1 bound. Increasing concentrations of thrombin passed over the SPR chip with the aptamer bound. Thrombin concentration increases from 0 nM, 5 nM, 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM. From injection the off-rates can be seen to be slow, highlighting thrombin:aptamer binding. The sensorgram is the response units of a channel containing the thrombin binding aptamer against a reference channel. Ruling out the possibility of non-specific binding interferring with the observed level of aptamer:thrombin interaction.

Using results extrapolated from the affinity plot of TBA v1 and v3 a distinct curve can be seen, following a classic michelis menten binding pattern, albeit on a reduced rate, a one to one binding for the interaction can be predicted. Although, this would be expected due to the competitive nature of the progressive iterations of SELEX that the aptamers were intially isolated using. An aptamer that required another oligonucleotide may not be selected as the amplification step would require both species to be amplified in equal measure, if both were to be amplified at all. The nature of PCR means that from the initial pool it is chance that dictates which sequences are amplified, a prevelence of one exact species rarely occurs and indeed, the are no reports of an aptamer requiring a binding partner to selectively bind it's

substrate. Also, when consensus sequences are drawn from a sequenced aptamer pool, two similar sequence could be grouped into a sub-group and following further investigation discarded due to an apparent lack of binding through no fault of the investigator.

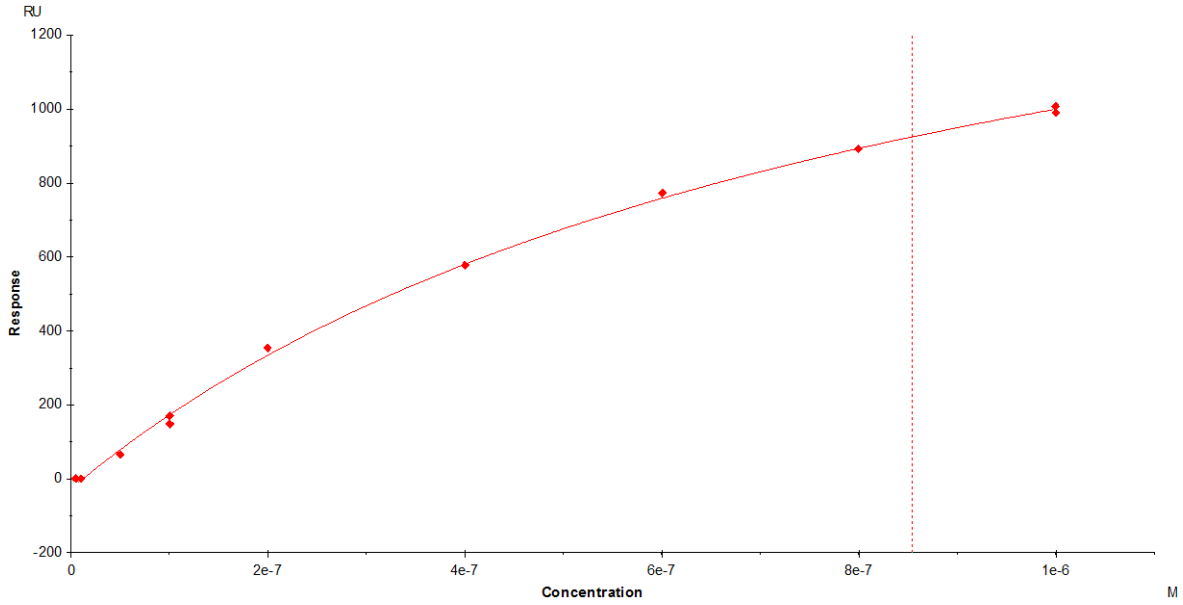


Figure 32 Affinity plot of increasing concentrations of thrombin binding to v1. Affinity plot of increasing concentrations of thrombin passed over surface bound aptamer v1. Graph created using BIAevalutaion software. Kd can be extrapolated due to estimated on/off rates gathered from the data.

3.5.3 Metal Ions

It has been reported in the literature that the G-quadraduplex that forms the three dimension motif requires a monovalent cation in order to form (Howard *et al.*, 1977, Clay & Gould, 2005). It has been proposed that for the thrombin binding aptamer this motif is held in place by a centrally localised K^+ ion with the presence of the potassium seen to be crucial for the formation of the structure (Dingley *et al.*, 2005). Using this prior knowledge of quadruplexes the fidelity of the technique can be assayed. The reliability up on the monovalent cation can be been attributed to the strong negative electrostatic potential that is created by the guanine's O6 oxygen atoms, which can be seen to form a central "channel" of the G-tetrad stack (Figure 33), with the cations located centrally within this "channel".

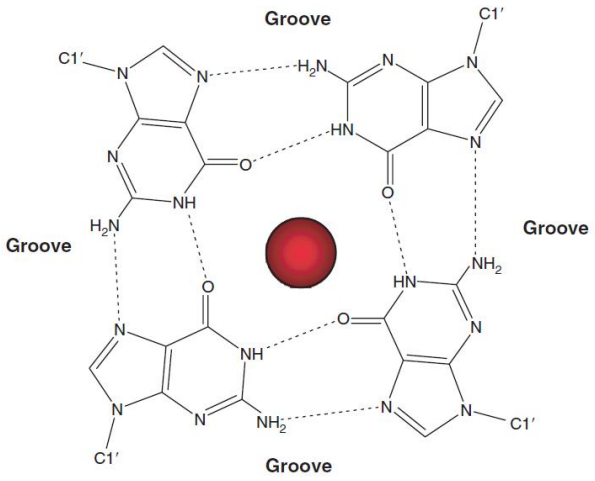


Figure 33 G-quadraduplex arranged around a bivalent metal ion. Schematic diagram of the formation of G-quadraduplex located around a centralised divalent metal ion (Burge et al., 2006)

As the cations are considered to be crucial for the formation and stability of TBAs secondary structure it is worth taking into account the role of other cations that may have a similar effect to the potassium. Na^+ for example can provide an alternative cation with monovalency as it is more commonly found in natural G-tetrads, but investigations have shown that the G-tetrad, when formed around different cations adopts a different topology depending on the element involved (Olsen *et al.*, 2009, Olsen *et al.*, 2006). This series of experiments was designed to investigate the role of the monovalent cation in thrombin binding aptamer affinity to thrombin, and to provide an insight into the nature of aptamer secondary structure using SPR.

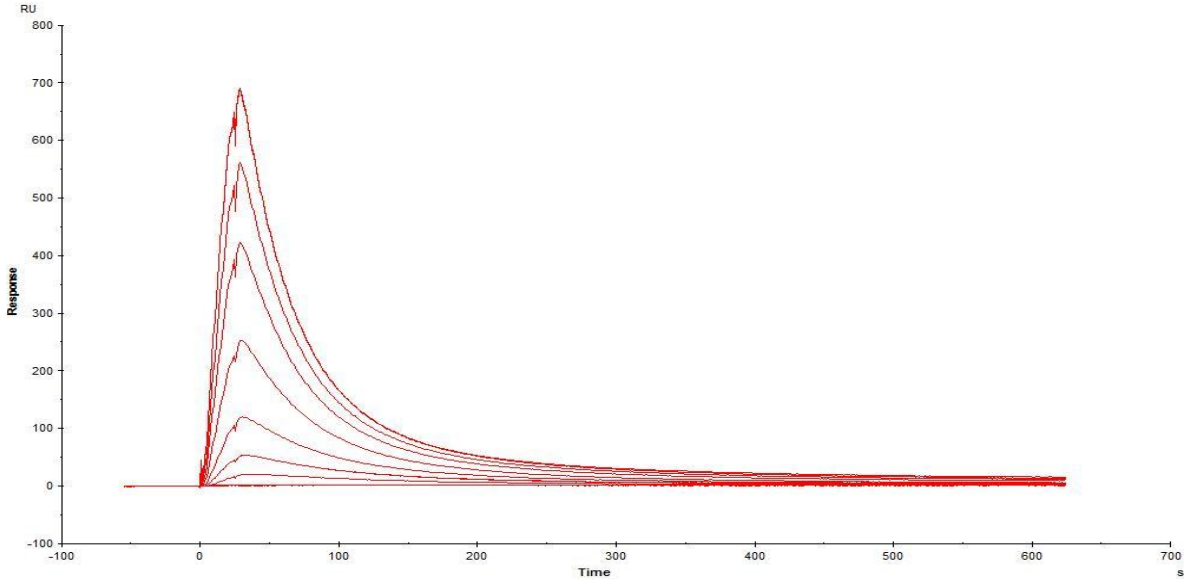


Figure 34 Sensorgram of thrombin binding aptamer to thrombin using the Lithium cation. Increasing concentrations of thrombin passed over the SPR chip with the aptamer bound. Thrombin concentration increases from 0 nM, 5 nM, 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM. The buffer varied from the regular running buffer by containing 50 mM Li.

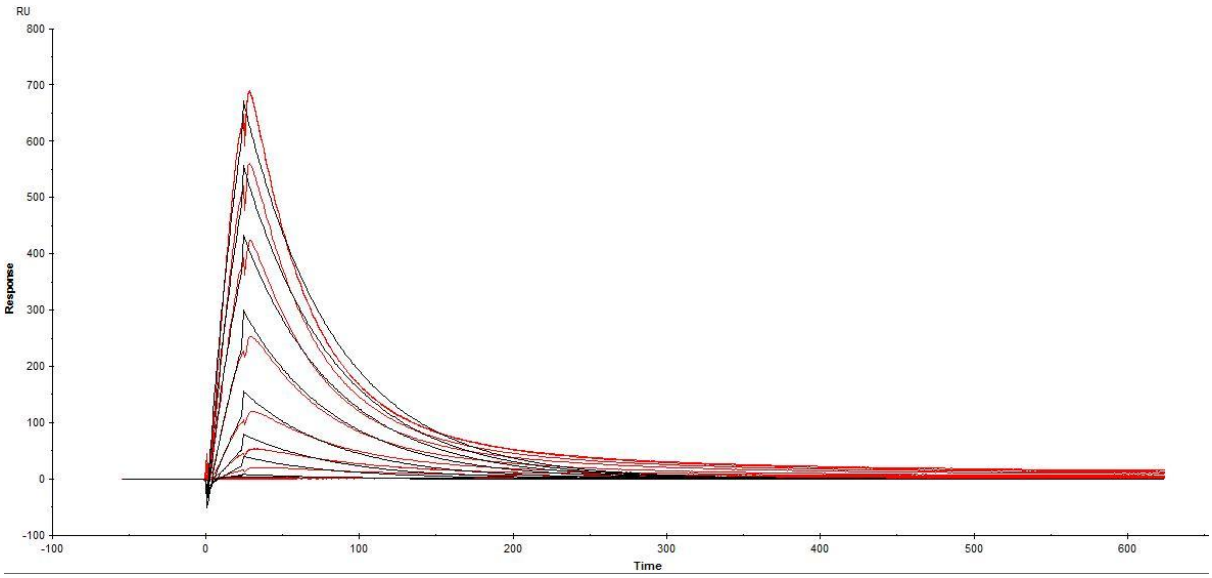


Figure 35 Sensorgram of thrombin binding aptamer to thrombin using the Lithium cation with a calculated fit. Reproduction of Figure 20Figure 34with fit calculated by BIAevaluation software according to a 1:1 binding stoichiometry

Table 5 is a summary of the data compiled from the thrombin binding aptamer to thrombin interaction as studied on the Biacore T100 Surface Plasmon Resonance machine (GE Healthcare®, Amersham, UK) with different monovalent cations present in the running buffer.

Table 5 Table depicting the apparent affinity constants derived from SPR data comparing different monovalent cations.

Thrombin Binding Aptamer variant	Monovalent Cation Apparent KD(M)		
	Li^+	Mn^+	K^+
v1	$3.55x10^{-5}$	$3.62x10^{-6}$	$8.5x10^{-7}$
v3	$8.79x10^{-6}$	$2.9x10^{-6}$	$1.5x10^{-6}$
R70E	$2.52x10^{-6}$	$1.5x10^{-5}$	$2.6x10^{-4}$

The data displayed in Table 5 provides a useful insight into the nature of binding of the thrombin binding aptamers when incubated in the presence of different monovalent cations. This would agree with the literature as discussed before as the aptamers and variants display a distinct increase in affinity for K^+ cations when compared to the estimated dissociation constants for the Li^+ and Mn^+ cations. This is in agreement with the literature (Kankia & Marky, 2001) which states the thrombin binding aptamer forms a more stable, and therefore, higher melting temperature complex with K^+ when compared with Li^+ and Mn^+. The proposed reason for this is that an ionic radius of 1.3-1.5 Å is required for the more stable G-quadruplex formation which the Li^+ and Mn^+ cations cannot bridge for because of their smaller ionic radii. In the same study it is suggested that Sr^+ can be a very useful replacement for K^+, but K^+ is a much more abundant cation, both biologically and scientifically.

3.5.4 Lysozyme control

In order to rule out non-specific binding of thrombin to the SPR chips or the aptamers, an identical control experiment using a similar protease, in this case lysozyme was used. Although lysozyme, a 14 kDa enzyme, is much smaller than thrombin it is a useful control because lysozyme can provide a removed null control that would demonstrate non-specific binding. As can be seen from the figure (Figure 36) the dissociation rates of the lysozyme are almost instantaneous but some non-specific binding can be observed. This is most likely due to the lysozyme interacting with the dextran matrix of the SPR chip as mentioned before and as a result the specificity of the aptamer can be determined with confidence.

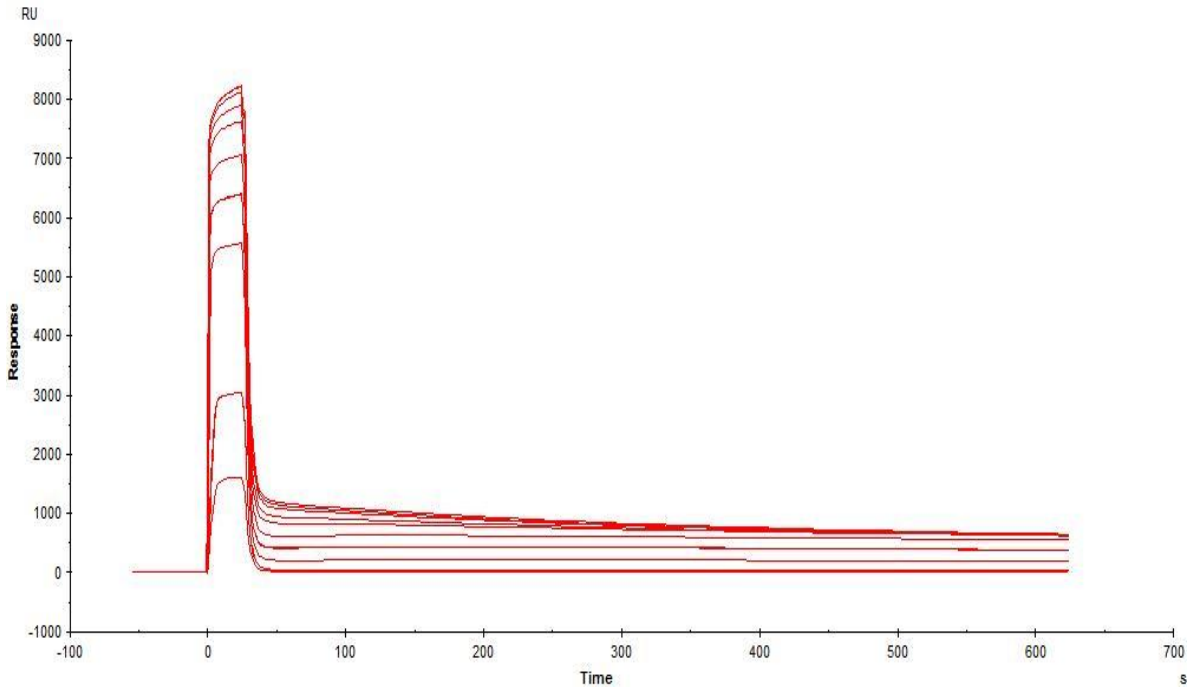


Figure 36 SPR sensorgram of the thrombin binding aptamer v1 when probed with increasing concentrations of lysozyme. Control experiment using lysozyme rather than thrombin highlighting the extent of specificity of aptamer to apitope. Identical conditions of aptamer, buffer, concentrations, contact times and flow rate but retention time minimal. Some non-specific binding can be observed, this is however, more likely to be interaction with the dextran matrix of the sensor chip not the aptamers.

3.5.5 Prothrombin

Thrombin forms the penultimate part of the blood clotting cascade, prothrombin is the precursor to the α-thrombin that has been the subject of the majority of this study to this point. Prothrombin is cleaved by Factor Xa to from the active serine protease thrombin, and it is through this action that the body can control the progression of blood clotting. In order to further investigate the thrombin binding aptamer and its interaction with α-thrombin it is useful to investigate whether binding is affected completely by the presence or absence of the part of the prothrombin (Bajaj *et al.*, 1981) precursor that is removed prior to the activation of thrombin. The experiment is the identical to the procedure that has been determined to provide reliable data and will probe the similarity between thrombin and prothrombin in relation to the thrombin binding aptamer, and give an insight into the specificity of the aptameric probes under investigation.

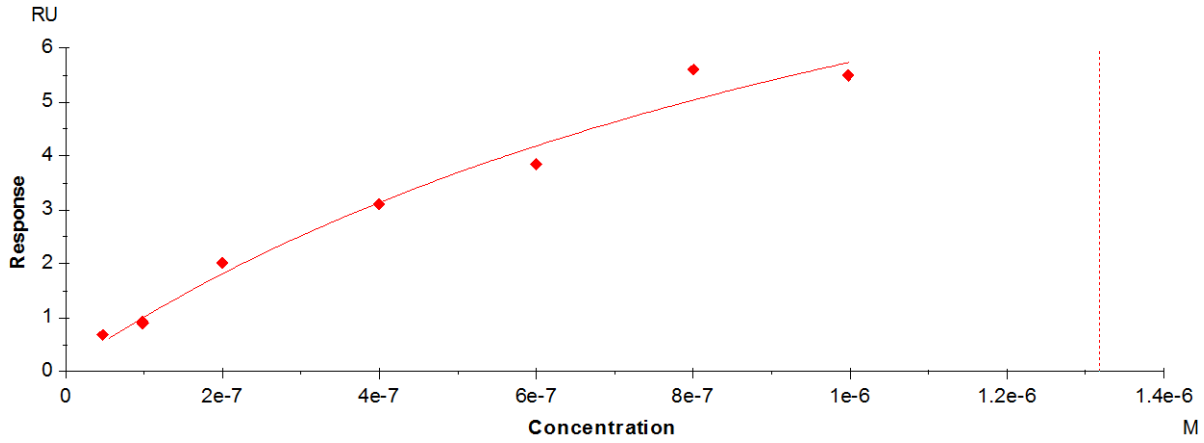


Figure 37 Affinity Graph of increasing concentrations of prothrombin binding to the thrombin binding aptamer v1. Affinity Graph of increasing concentrations of prothrombin binding to the thrombin binding aptamer, sensorgram produce on the Biacore T100 SPR machine using identical parameters to the standard experiment derived earlier.

The apparent dissociation constant of the Prothrombin interaction with TBA v1 is according to the information recovered from SPR data and converted to an approximate value. Using the projected concentration graph it can be seen that the dissociation constant where $K_a=K_d$ would be at around 1.34×10^{-6}. This apparent dissociation constant must be treated with some trepidation however, as the response units for the interaction are low (when compared to the average response units for the same interaction using thrombin which ranges from 150-200 RU for the higher concentrations). As the response units are so low it can be assumed that the results for this interaction can largely be ignored, as although there is some binding, it is of little to no consequence and can be attributed to non-specific binding. The sensorgram from which this data is drawn can be seen to serve the argument that the binding is restricted to non-specific events although it is not included here.

3.5.6 Assembly and disassembly

A good biosensor can be said to be able to reliably and sensitively detect specific biological with a high signal to noise ratio. A challenge to the creation of a biosensor is the ability to interchange the target molecule without the need to completely rebuild an entire system. Aptamer technology may provide a useful method of change the analyte of interest because of the relative ease with which DNA can be constructed, deconstructed and clean within a laboratory environment. It is proposed that an anchor region of a complex can be immobilised on a surface by biotin:streptavidin interaction, covalent cross-linking of the phosphate backbone, or thiolation of an activated group attached during synthesis. To this

single stranded anchor portion of DNA an aptamer can then simply be annealed through interaction of the Watson and Crick base pairing of a complementary segment that is 2-6 base pairs in length. Using the previously designed and characterised thrombin binding aptamer:thrombin binding partner's experiment using Surface Plasmon Resonance, the possibility of using a system derived from this can be explored.

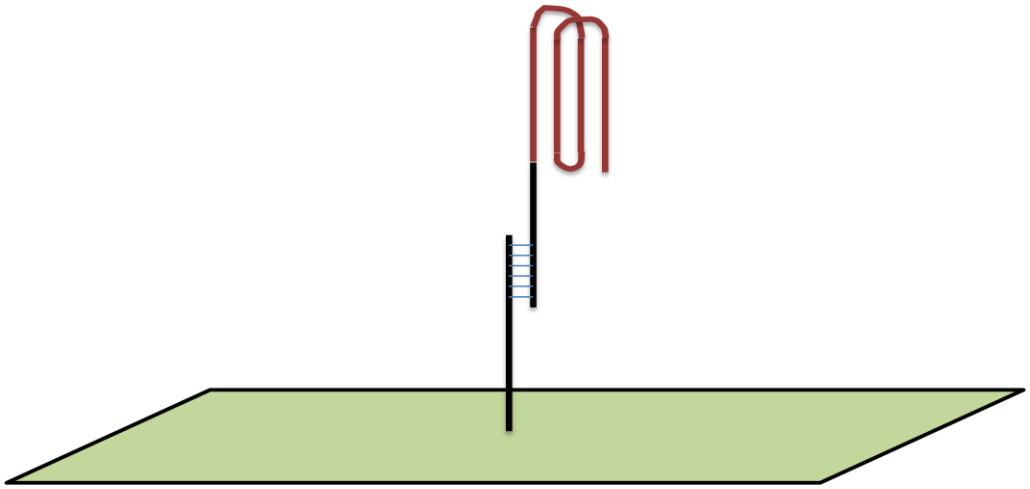


Figure 38 Schematic diagram of the thrombin binding aptamer TBA v1 attached to anchor region immobilised to an SPR chip via biotin streptavidin interaction. A cartoon representation of the construction of the surface bound aptamer assembly. The surface is a streptavidin coated dextran matrix to which the 5' biotinylated DNA anchor region is bound with high (15 fM) affinity. The blue represents the complementary binding region of varying length, with the TBA v1 portion represented in red.

To localise the anchor region to the streptavidin coated dextran matrix of the SPR chip the 5' terminal nucleotide of the anchor sequence has a biotin attached during synthesis. The anchor region sequence is as follows: 5'biotin-GGCCTATATAGCGC 3'. The previously defined Thrombin Binding Aptamer Sequence: GGTTGGTGTGGTTGG (Bock et al., 1992) was synthesised with increasing regions of complementarity as detailed in (Table 6) **.** As the number of complementary base pairs increases the energy required to remove the hydrogen bonds formed between the bases will increase. The increase of dissociation energy will serve to stabilise the two DNA strands, in theory, as the length of the overlapping section increases the higher the ionic strength of the buffer required to dissociate the TBA v1 from the immobilised anchor.

Table 6 **Table detailing the construction of Thrombin binding aptamer to anchor conjugates**

Complementary Region Length	Anchor Region	Thrombin Binding aptamer with overlapping region	Overlapping Region Tm (kcal/mole)
1 base pair	5'biotin-GGCCTATATAGCGC 3'	3' GTAGGTTGGTGTGGTTGG 5'	-1.5
2 base pairs	5'biotin-GGCCTATATAGCGC 3'	3' CGTAGGTTGGTGTGGTTGG 5'	-3.07
3bp base pairs	5'biotin-GGCCTATATAGCGC 3'	3' GCGTAGGTTGGTGTGGTTGG 5'	-6.75
4bp base pairs	5'biotin-GGCCTATATAGCGC 3'	3' CGCGTAGGTTGGTGTGGTTGG 5'	-9.89
5bp base pairs	5'biotin-GGCCTATATAGCGC 3'	3' TCGCGTAGGTTGGTGTGGTTGG 5'	-11.49
6bp base pairs	5'biotin-GGCCTATATAGCGC 3'	3' ATCGCGTAGGTTGGTGTGGTTGG 5'	-12.45

For ease of study three overlapping regions were selected of 2, 4, and, 6 base pair overlaps for comparison. The 2 base pair overlap would be the least stable of the three with the 6 base pair overlap comparatively stable and the 4 base pair overlap exactly halfway when considering stability. Conversely, the least stable binding partners should be the easier to reassemble following denaturation, due to the lower amount of energy required to form the hydrogen bonds between the pairings.

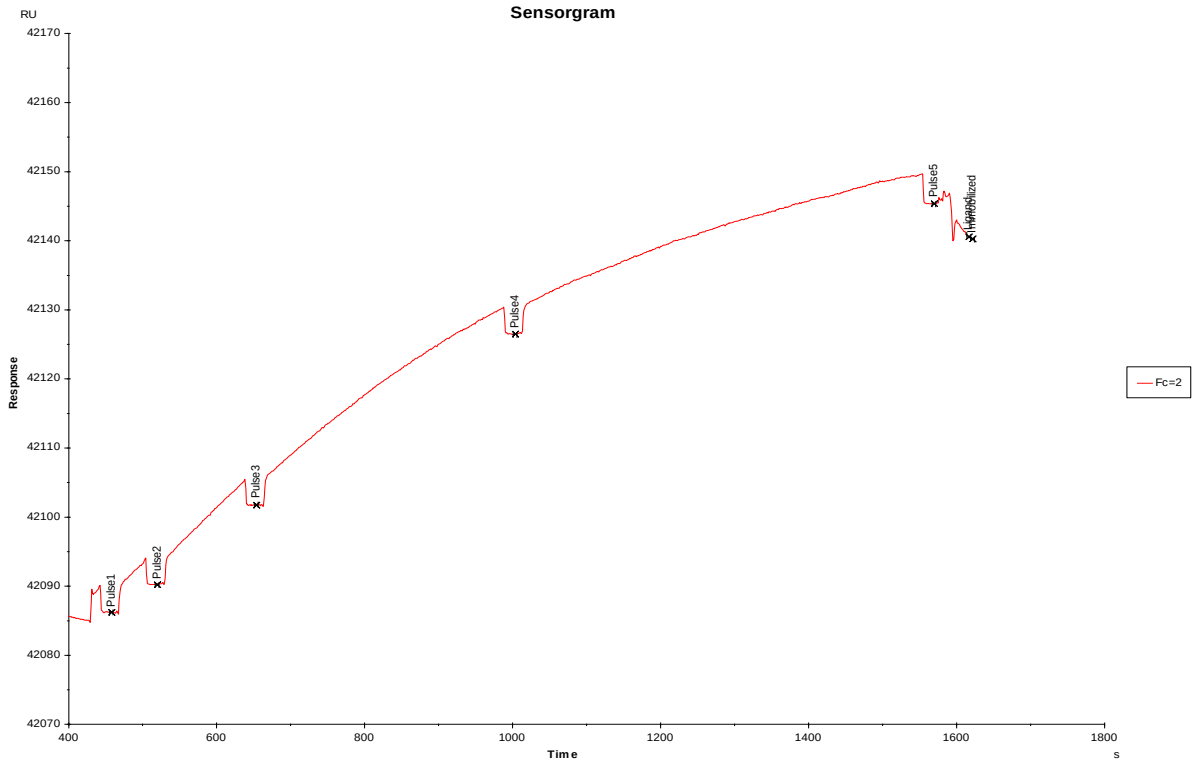


Figure 39 Overlapping Region OLR 2 immobilised to the SA chip. Overlapping Region OLR 2 immobilised to the SA chip. Sequential pulses of biotinylated aptamer anchor conjugate Overlapping Region OLR 2 immobilised to the Streptavidin coated SPR chip

Following the successful immobilisation of the aptamer:anchor conjugate to the SPR chip the validity of the thrombin binding portion of the aptamer was tested for comparison to the unmodified thrombin binding aptamer v1 tested previously. Identical conditions were used for determining the viability of the annealed aptamer to those used to determine the binding conditions and kinetics of the thrombin binding aptamer v1. The resulting sensorgram (Figure 40) of this assay will give an insight into whether this method can be considered for the construction of an interchangeable molecular recognition element of a biosensor.

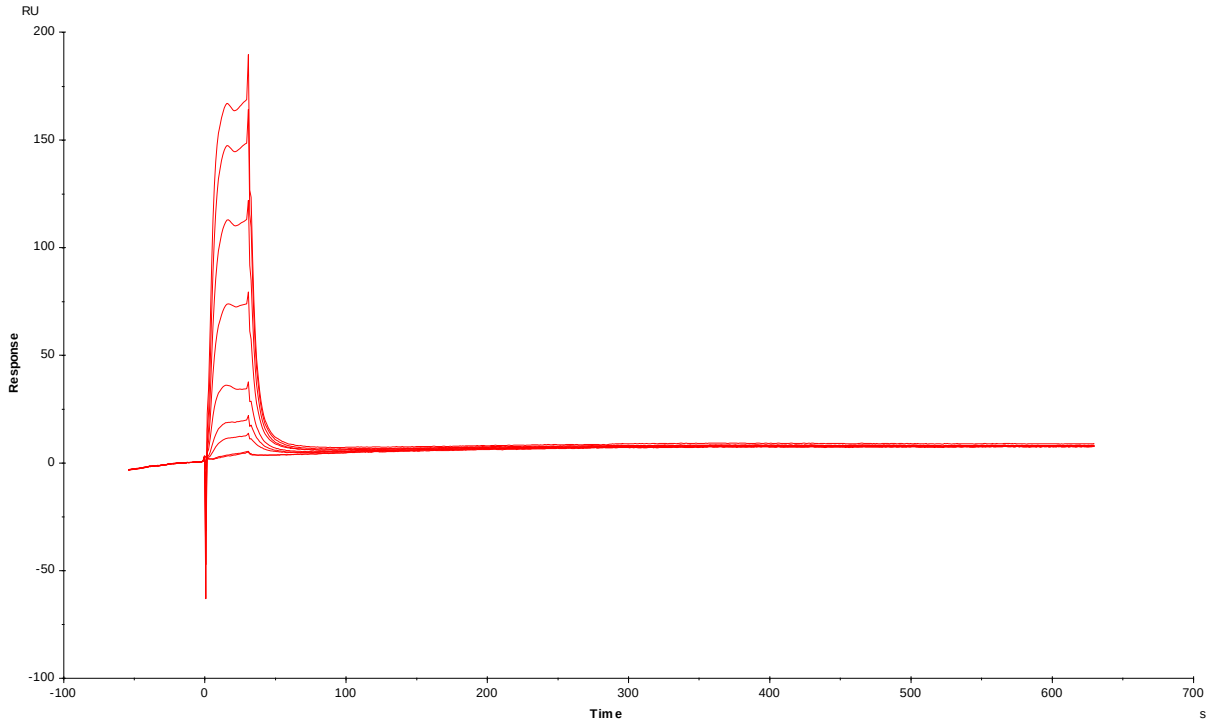


Figure 40 Increasing concentrations of thrombin passed over TBA v1 annealed to the anchor region. Sensorgram of increasing concentrations of thrombin interacting with TBA v1 attached by annealing to anchor region v1. Sensorgram created by Biacore T100 Surface Plasmon Resonance machine.

Following the successful immobilisation of the aptamer:anchor conjugate to the SPR chip the validity of the thrombin binding portion of the aptamer was tested for comparison to the unmodified thrombin binding aptamer v1 tested previously. Identical conditions were used for determining the viability of the annealed aptamer to those used to determine the binding conditions and kinetics of the thrombin binding aptamer v1. The resulting sensorgram (Figure 40) of this assay will give an insight into whether this method can be considered for the construction of an interchangeable molecular recognition element of a biosensor.

As can be seen by the sensorgram (Figure 40) the thrombin does interact in a concentration dependent manner, indicating that the aptamer is constructed and located on the chip after successful attachment and creation of the complex. A challenge remains as to whether the formation, can then be deconstructed, and then re-assembled within the environment required for accurate generation of results. Figure 41 is an adjusted version of the sensorgram Figure 40 with a calculated fit assumed for the projection of binding kinetics as determined by the program BIAevaluation (GE Healthcare, Amersham, UK).

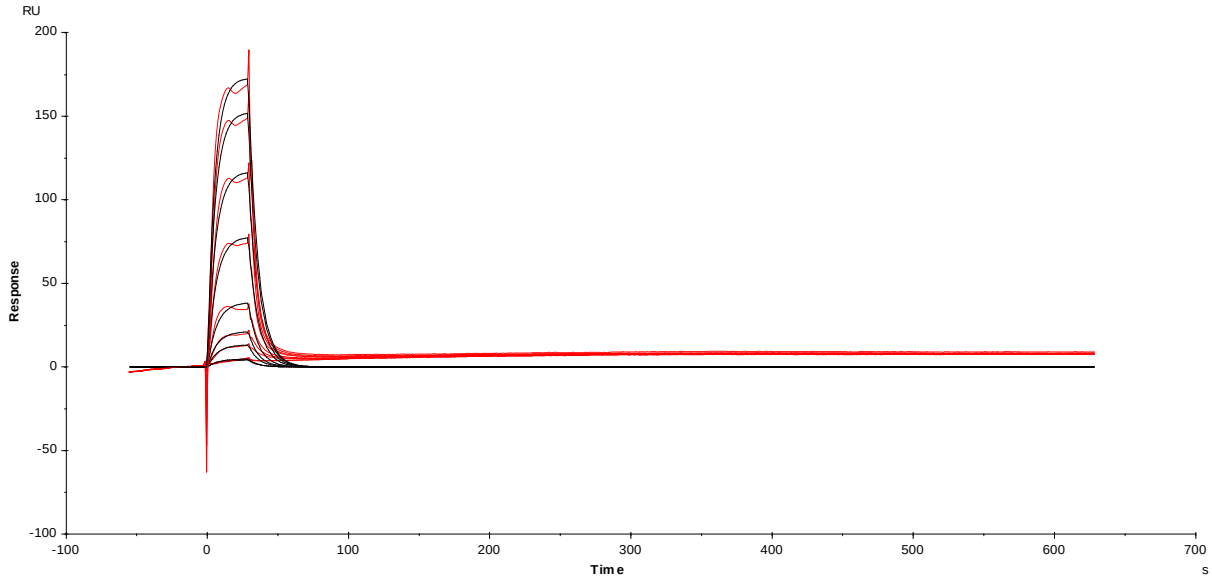


Figure 41 Sensorgram depicting thrombin interacting with OLR aptamer with calculated fit. Sensorgram depicting thrombin interacting with OLR aptamer with calculated fit. Sensorgram details increasing concentrations of thrombin interacting with TBA v1 attached by annealing to anchor region v1. Sensorgram created by Biacore T100 Surface Plasmon Resonance machine.

Following the successful immobilisation of the aptamer:anchor conjugate to the SPR chip the validity of the thrombin binding portion of the aptamer was tested for comparison to the unmodified thrombin binding aptamer v1 tested previously. Identical conditions were used for determining the viability of the annealed aptamer to those used to determine the binding conditions and kinetics of the thrombin binding aptamer v1. The resulting sensorgram (Figure 40) of this assay will give an insight into whether this method can be considered for the construction of an interchangeable molecular recognition element of a biosensor.

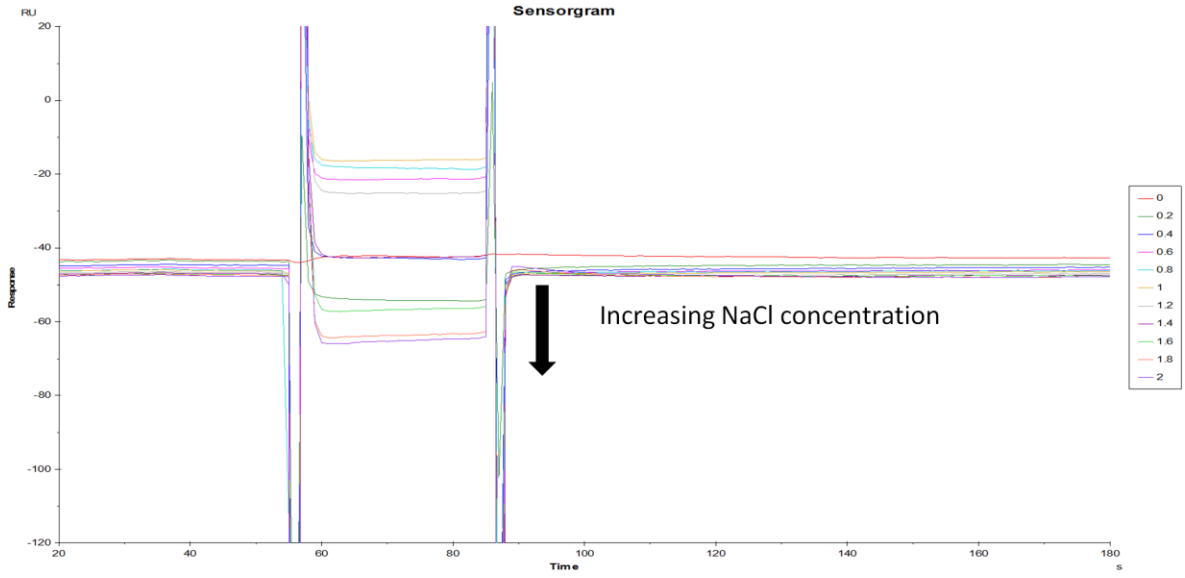


Figure 42 Sensorgram displaying increasing concentrations of NaCl affecting RU for the OLR Aptamer. Sensorgram displaying increasing concentrations of NaCl affecting RU for the OLR Aptamer. This sensorgram depicts a reduction in SPR response units following the application of the NaCl washes

As can be seen from the sensorgram (Figure 42) the first sample of running buffer (in red) has the expected result of no discernable response unit change, but, from 0.2 M NaCl through to 2 M NaCl the response units drop following each round. This change can be attributed to the OLR of the aptamer that is attached to the anchor being dissociated through a higher ionic strength buffer overcoming the energy needed to keep the DNA base pairs together. The immobilised anchor regions were then subjected to the pre-defined thrombin binding kinetic program and found to not bind the thrombin above a level associated with non-specific binding. From these results it can be assumed that the thrombin binding aptamer portion of the conjugate has been displaced, but, the initial goal of the experiment was to displace and then re-assemble the complex when immobilised to the chip. This proved extremely difficult due to the limitations of the machine on which the assays were to be completed which prevented the incubation as the flow rate can only be slowed, and not halted.

3.6 Conclusions

The concept for the cleavage assay remains solid, but, further investigation or alternative equipment specifications may show that the ratio of signal to noise could be pushed to a more favourable conclusion. A further complication is the apparent half-life of the peptide substrate. This was found to be far below an acceptable level; given the relative cost of

synthesising the substrate such a poor return renders the consideration of this method as a viable, long-term and repeatable assay questionable. The data acquired from the assay however, can be seen to support that which has previously been published on the nature of the Thrombin Binding Aptamer and its affinity to the substrate.

Some sound conclusions can be drawn from the Surface Plasmon Resonance experiments however; the technique gave consistent, comparable, and reproducible data regarding the determination of a dissociation constant for the TBA species and their target protein thrombin. The findings also served to strengthen data supporting the published conclusions of specificity regarding the aptamers and thrombin. By using the precursor Prothrombin and the unrelated protein lysozyme as controls for non-specific binding it can be said that TBA v1 and v3 will bind specifically thrombin and that the binding site is interfered with by the peptide “shield” of the fibrinogen binding active site. This secondary conclusion supports the initial findings in the original paper of Bock *et al* ((Bock et al., 1992) in which the aptamer pool was selected against coagulation rates of blood. Drawing meaningful and convincing conclusion from the Surface Plasmon Resonance data remains complicated. This is due to the fact that directly comparing the binding affinities of interactions between molecules continues to provide a number of difficulties because of the nature of the technique only showing shape changes when associated with the chip surface. As referred to earlier, one drawback of Surface Plasmon Resonance is the determination of mass of the molecule interacting with the surface when the technique is limited to detecting the shape of interfering molecules.

As the concept driving this chapter is the determination of a suitable method for a rapid, reliable, and reproducible method for the assay of aptamer to target interactions, there must be some consideration as to the length of time needed to create such a method using SPR. The process of repeated and expensive iterations with the intention of arriving at an assay can be a time-consuming one. Once a concept for the reaction conditions required to begin such a labourious task has been determined, the systematic process of repeating the protocol with slight alterations can cause deterioration of the expensive specialised chips after time.

The model for the building and deconstruction of an aptamer scaffold is an attractive one as it provides the means to alter the "meta" biosensor in a cheap and rapid way. Attaching an aptamer to a scaffold creates a modular approach to aptamer characterisation allowing the interchanging of aptamers without the need to redesign. The method has been shown to work as far as the assembly of the anchor-aptamer scaffold to the removal of the active aptamer portion. Replacing one aptamer for another without the need to set up an entire experiment increases the throughput of an assay greatly, as aptamer generation methods can produce large amounts of possible aptamer sequences which need to be validated. If the method can be automated then the prospect of an interchangeable aptamer recognition element remains a seductive one, however, this investigation into the viability of the reusable portion of a biosensor stalled at the regeneration of the conjugate. Due to low dissociation constant of the streptavidin to biotin binding interaction, this rules out regeneration of the surface with an entirely new aptamer:anchor conjugate or novel aptamer species, the only method that was forthcoming was the removal and replacement of the active aptamer. Whether the removal is through an high ionic strength buffer, such as 2-3 M NaCl, or the use of a restriction enzyme that would cleave the DNA at a predefined point within the sequence of the anchor using the Biacore T100 SPR is restrictive in the available conditions for the annealing of any single stranded DNA to the immobilised anchor region. As the over-lapping region of the two strands varied from 2 to 6 base pairs, it could be assumed that the temperature required for the formation of the hydrogen bonds necessary to create the base could be achieved. Each base pair requires an increase of temperature of approximately 6^{o}C, so it can be assumed that the 2 base pair over-lapping region would require around 12 ^{o}C to form, 24 ^{o}C for the 4 base pair overlap and 36 ^{o}C for the 6 base pair overlapping region. Although, the machine was capable of achieving the temperatures required to ensure annealing of the complementary regions, the program prevents the user from pausing the flow of sample on the chip. This is potentially what prevented the two binding partners to form the scaffold, as no discernable response units increase was observable. The repetition of the thrombin binding protocol helped to confirm the summation that the scaffold had not re-assembled because there was a distinct lack of interaction between thrombin and the oligonucleotide immobilised on the surface above that of non-specific binding.

4 Production of novel aptamers – Immobilisation methods

4.1 The HsdR subunit of the Type II Restriction Modification system EcoR124I

In this chapter the method of affinity SELEX is undertaken, with the aim of producing novel aptamers to two distinct protein targets, GST-HsdR and GST-HRV143C(GE Healthcare). Both protein targets have a glutathione-S-Transferase fusion protein affinity tag and the SELEX iterations carried out using glutathione sepharose as the affinity media. The entire screening process is carried out using a bench top microcentrifuge, highlighting the ease with which SELEX can be achieved. The intention of the characterisation of the method is to provide a quick, reliable and simple method to isolate aptamer species that could be undertaken by any user. Once the aptamer pools have been defined the next step is to characterise those species of interest, and methods need to be identified with which the potential aptamers can be characterised and some work is done towards determining the most useful of the potential aptamers. Using the methods already described earlier such as Surface Plasmon Resonance assays, there is an attempt to characterise the binding of the aptamers to the target with the aim of determining a dissociation constant that can be associated with each species. Any aptamers arising from the selection will be good candidates for use within the generic biosensor as a molecular recognition element.

HsdR is the largest individual subunit of the type I R-M enzymes at 120 kDa and associates with the MTase to produce the restriction nuclease (Yuan, 1981).The HsdR subunit of the type I restriction modification enzymes is required for DNA cleavage and translocation when complexed with the MTase complex forming the endonuclease. HsdR has not been shown to have any independent activity through DNA binding experiments (Zinkevich *et al.*, 1997) however it is responsible for ATPase activity of the endonuclease which has been shown only in the presence of the HsdR protein. This ATPase activity has been linked to a motor activity, which allows the translocation of DNA through the DNA-bound enzyme (Davies *et al.*, 1999b, Davies *et al.*, 1999a), unidirectionally and independently for each HsdR subunit, along polymer tracks (Kliche *et al.*, 2001). The translocation activity is similar to helicase activity and identified to contain super family two helicase motifs through sequence alignment of the DEAD/H box motifs (Gorbalenya & Koonin, 1991)

The main activity of the HsdR subunit is the restriction activity. HsdR binds with the MTase complex and switches to a restriction enzyme on the detection of un-methylated DNA at the restriction site (Yuan & Meselson, 1970). The restriction-modification enzyme has been shown to remain attached to its recognition site resulting in DNA being looped beneath the molecule. DNA cleavage takes place when translocation is impeded either by collision with another translocating complex or by the topology of the DNA substrate. Combined, these factors make HsdR a good candidate as a target for aptamer selection, being a well characterised protein helps to optimise the selection and later aptamer validation towards methods known to work with the native protein.

4.2 HsdR SELEX

The HsdR subunit of EcoR124I has a definitive role in the cleavage of double stranded DNA, providing a primitive type of immune system for *E. coli* when complexed with the co-expression partner subunits, HsdS (the specificity subunit) and HsdM (the methylation subunit). Complexed together the subunits form a restriction enzyme that identifies hemi-methylated foreign DNA which it translocates and cleaves at two un-specified points. It is the HsdR subunit which is responsible for the translocation of the DNA (Weiserová & Firman, 1998) and it has been shown to be an ATP dependent (Davies et al., 1999a) process. It is because of the ability of the HsdR subunit to translocate DNA that the subunit can be said to be a molecular motor "pulling" DNA through it, as opposed to moving along in a ratchet type manner.

Due to the interest in the HsdR subunit as a crucial part of a generic biosensing system, it was an obvious choice for the first target to which aptamers were to be isolated. Crucially, the HsdR gene has previously been cloned into a plasmid vector that, when expressed as a protein, gave rise to a fusion protein. The fusion protein consists of a 26000 Da fusion protein Glutathione-S-Transferase (GST) then a peptide linking region containing a cleavage site (for Human Rhinovirus 14 3C or (HRV143C) which is then followed by HsdR subunit. The addition of the GST provides a means with which to bind the protein to a column through the use of Glutathione Sepharose media (GE Healthcare®, Amersham, UK). The interaction of the GST with the glutathione sepharose can be said to be relatively strong with a dissociation constant in the region of K_d = 6.9 X 10^{-9} mol/L (Waterboer *et al.*, 2005), the binding of the two partners has been utilised within affinity chromatography to purify

proteins. It is proposed that through the immobilisation of the target via the GST:Glutathione sepharose interaction to the column it will be possible to isolate a pool of aptamers to the HsdR subunit of EcoR124I using SELEX.

If the protein can be said to be reversibly attached to the column media, the aptamer library can be introduced to the bound protein and following thorough washing any bound species can be collected by elution with a high ionic strength buffer. Some attention must be paid to the removal of potential aptamers species that will bind to either the glutathione sepharose media or to the GST portion of the fusion protein. These un-wanted sequences are removed by a pre-screening step that involved the introduction of the library to the cleaved and washed GST and remaining linking region bound to the column. Any species that do not bind to the media or any unwanted portion of the apparatus are removed using this step. Once regenerated following a series of washes using a glutathione containing buffer the complete fusion protein of GST-HsdR is bound to the column and the first round of SELEX screening is undertaken. Following 13 rounds of sequential screening and amplification the pool of potential aptamers is cloned into a sequencing vector and sequenced using Sanger sequencing. Following sequencing, the library of putative aptamer sequences can then be probed to determine whether an aptamer with high specificity and affinity has been isolated or a consensus sequence for the reliable binding of the aptamer to the protein has been arrived at.

Although GST-HsdR has Glutathione-S-Transferase attached to the target, there are other methods to reversibly immobilise a protein target to affinity media. There are a number of commercially available affinity tags such as HIS, CBP, CYD (peptide tag), Strep II, FLAG, HPC (heavy chain of protein C) peptide tags, MBP a second protein fusion tag (Lichty *et al.*, 2005). Each method provides a compromise between high affinity resin and low binding capacity, when purifying proteins. If, as in the case of SELEX the process requires previously purified protein samples with the affinity tag in place, the binding capacity is not relevant but, the affinity of the resin is. This is due to the fact the lower the concentration of the target which the library of random oligonucleotides is applied to the more stringent the SELEX round will be. As there is less physical material for the different species of potential aptamers to bind to, the species with the higher affinity will out-compete those lower affinity aptamers making it more likely that the surviving species will be selected.

4.3 HsdR SELEX

The progressive rounds of SELEX can be seen in Figure 43, this native PAGE gel can be used to follow the refinement of an aptamer selection library. A distinctive band can be seen at 75 base pairs in length. Although the library should be 54 base pairs in length this change in migration difference can be attributed to the secondary structure formation of the aptamer species causing retardation through the acrylamide gel matrix. Each sample is taken after the amplification step of each round and can be assumed to not be PCR primers because of comparison with the first lane of just the 1 µM forward and reverse primers used in the amplification of the SELEX library.

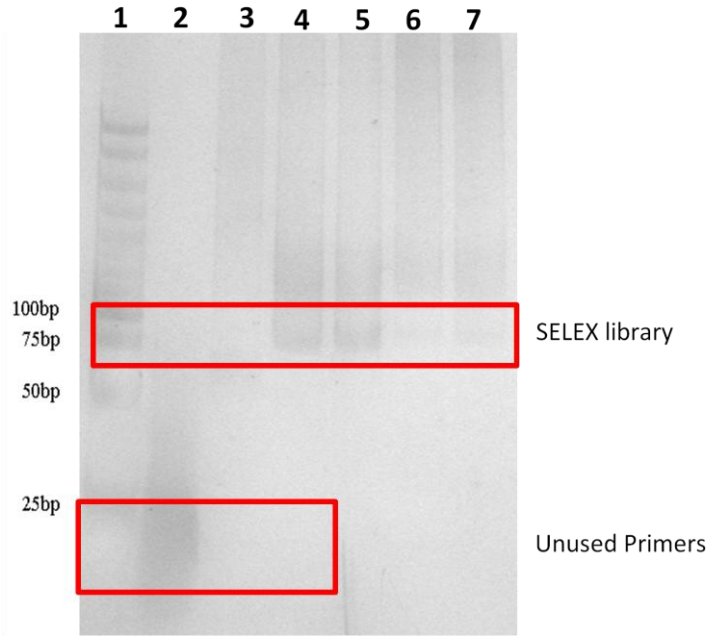


Figure 43 Progressive rounds of SELEX to HsdR showing the development of aptamer pools. 16% Native acrylamide gel. Lane 1 Hyperladder V (Bioline), lane 2 unused primers, lanes 3,4,5,6,7,are rounds 9,10,11,12,13 of SELEX respectively. Stained with Silver stain (FLUKA).

Figure 44 depicts a detailed insight into the final aptamer pool before insertion into a cloning vector. This shows the aptamer library at closer to its true length of 50 base pairs in length and also highlights some impurities from possible mis-priming occurring in the amplification process.

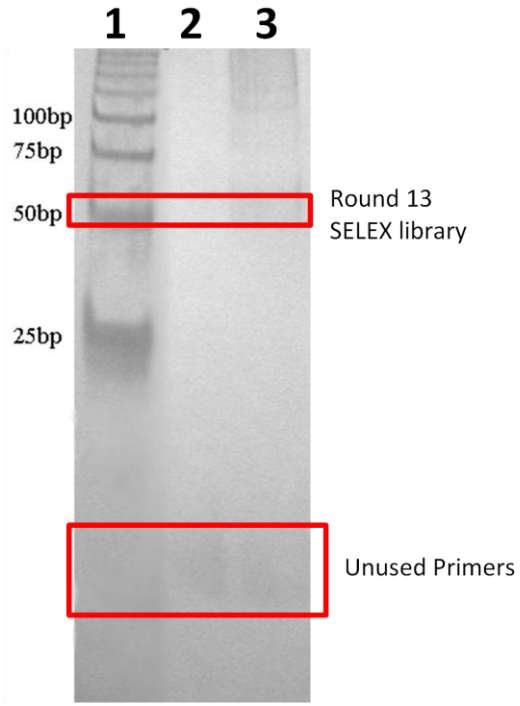


Figure 44 Native Gel showing the final pool before cloning into pGEM-T-Easy. 16% native acrylamide gel. Lane 1 Hyperladder V (Bioline), lane 2 unused primers, lane 3 is result of round 13 PCR amplification. Stained with Silver stain (FLUKA).

After the full length of 13 rounds of SELEX screening and amplification the final screened library is cloned into a vector that utilises the fact that during polymerisation *Taq* DNA polymerase will leave an adenosine at the terminus of polymerisation. The pGEM-T-Easy™ (Promega) vector is linearised with a final thymidine overhang which eases the ligation of two DNA segments. As the only requirement for the insert is the automatic A-overhang left by polymerisation with a common polymerase, the preparation of the aptamer library for cloning is simple and rapid, as once the plasmid is cloned into a host bacterium the amplification of the potential cloned library is rapid and effective as it is carried out through the life cycle of the bacteria. After selection for a single colony, each potentially successful clone is extracted from the cells and prepared for sequencing that is carried out by Cogenics®. One potential area of error in cloning using this method is the orientation, and number of potential inserts within the vector. As the clones can orientate in any direction, some care needs to be taken when studying the resultant sequences and the direction of the primers needs to be established to determine whether the putative aptamer is viable.

Table 7 List of potential HsdR binding aptamers

Aptamer name	Aptamer Sequence	%C	%G	%A	%T
HBA 1	CCAAGCGGGCGCGAGAACAACGTACGGT	28.5%	35.7%	28.6%	7.1%
HBA3	CCGAGCAGAAGGTGCGATGCTTTATGGT	14.3%	35.7%	21.4%	25.0%
HBA8	CCGAGCAGAAGGTGCGATGCTTTATGGT	10.7%	35.7%	21.4%	25.0%
HBA14	CCCGGGCGGACAGACGGGTGTTCGTTGC	28.6%	42.8%	10.7%	17.9%
HBA15	CCCGGGCGGACAGCAGGCTGTTCGTTGC	32.1%	39.3%	10.7%	17.9%
HBA16	CCCGTAGCGTACAGTCCCAGCTTGGGAT	32.1%	28.6%	17.9%	21.4%
HBA20	CCACAACGAGCCGGGATTGCGGATTGCA	28.6%	32.1%	25.0%	14.3%

Table 1 shows the results of the putative aptamers recovered from sequencing of the putative aptamer clones. The sequencing results gave 7 potential sequences for further characterisation in order to determine affinity and specificity for each individual species. An interesting note is the propensity for a majority of guanine residues, this hints at the formation of the g-quadraduplex motif.

4.3.1 Non-denaturing High Performance Liquid Chromatography of Progressive rounds of HsdR SELEX

As the iterative process of aptamer pool refinement progresses it is difficult to ascertain the development of definitive groups of binding motifs or the clonal dominance of some sequences when compared to the co-isolated library without time consuming and potentially distracting investigations into the nature of the library at each round throughout the refinement. Using HPLC provides an insight into the development of the enriched library.

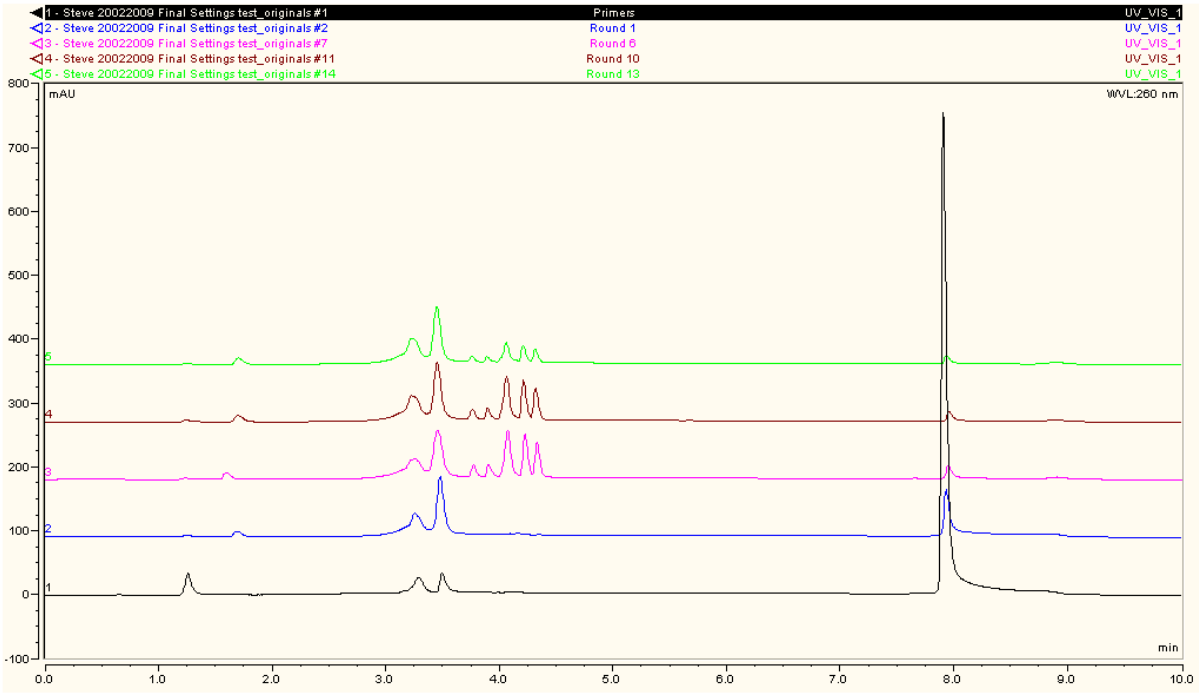


Figure 45 Non-denaturing HPLC analysis of the refinement of HsdR aptamer library. Chromatograms of Progressive rounds of HsdR SELEX analysed using the Dionex 3000 HPLC system and DNAPac column. The peaks symbolize the absorption of UV light at λ260 nm and represent the refinement of the DNA aptamer library through rounds 1,6,10, and, 13 of HsdR SELEX.

Using non-denaturing High Performance Liquid Chromatography methods is possible to see the refinement of the aptamer library into three distinct groups. As the method does not denature the secondary structures the putative aptamers form the different groups will migrate through the resin at different rates, due to simple occlusion of the motifs. As each round of SELEX progresses there are three well defined peaks, each indicating a different motif with two smaller peaks that may be the species that are not selected against.

4.3.2 HsdR binding

Once the potential aptamers were recovered from the received sequencing data an initial experiment to determine the viability of the aptamers was undertaken. This simple and cost-effective method relies on the protein adhering to a nitrocellulose membrane which is then blocked with Bovine Serum Albumin (BSA), to prevent non-specific binding of the biotinylated aptamer probes. The aptamer to antigen interaction can then be probed using a Horse Radish Peroxidase:streptavidin conjugate (HRP) as a reporter molecule, as this will bind to the biotin of the aptamers. An insight into the viability of the aptamers species can be gained by application of a constant concentration of aptamer to decreasing immobilised target concentrations. Using this method it is also possible to determine putative

dissociation constants for aptamers quickly and in a multiplex fashion. It is also advantageous to probe specificity in the assay by including a control protein, such as lysozyme.

4.3.3 Electrophoretic Mobility Shift Assay of HsdR and potential aptamer candidates

A well established and common practise for the first investigations for probing a suspected binding interaction is the Electrophoretic Mobility Shift Assay (EMSA). The components of the binding assay are incubated together and subjected to an electric field inducing them to move through a gel matrix which will separate molecules according to size. When in complex with each other the larger ligand:target will migrate through the matrix at a slower rate than the constituents parts. The technique can be sensitive enough to provide data "in bulk" that can be extrapolated into dissociation constants in addition to other binding information such as whether a protein dimerises in solution, or perhaps, the candidates require trimerisation for example. The nature of the technique renders it a cost effective and rapid method for high-throughput screening of multiple candidates simultaneously.

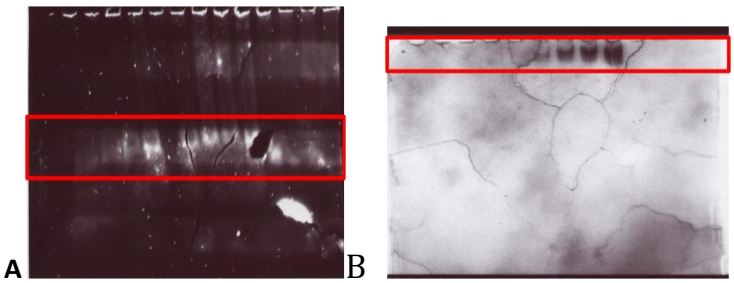


Figure 46 Electrophoretic Mobility Shift Assay of increasing concentrations of HsdR incubated with 10 µM HBA15. EMSA depicting the binding of HsdR with the aptamer ligand HBA 15 in a 6% Native acrylamide gel. HsdR concentrations going from left to right 0, 10nM, 50 nM, 100 nM, 500 nM, 1 µM, 2.5 µM, 5 µM, and 7 µM. The gel is first stained with SYBR gold nucleic acid stain (A) then with Coomassie Brilliant blue protein stains (B).

Figure 46 binding can be observed for HBA15 from approximately 500 nM through to 7 µM, and although this does give a positive result as to whether the aptamer in question does bind or not, the data recovered is not sensitive enough to carry forward for further manipulation. The bands forming can be attributed to HBA 15 binding to HsdR as retro Coomassie blue denaturing staining of the gel indicates the location of the HsdR band's

migration into the gel, corroborating the findings that the aptamer is binding to the target in a bulk solution. Another method for the validation of aptamer binding was sought for greater certainty and clarity for the characterisation of the HBA to HsdR binding partners.

4.3.4 Dot blots

Dot blots are a quick and useful technique for the analysis of two binding partners. In this method increasing "dots" of protein are bound to nitrocellulose membrane and allowed to dry. A solution of the second binding partner is washed over and binding is assayed through a reporter attached to the second binding partner (Materials and Methods 2.10)

The initial prospective dot blots used a micro molar range of concentrations to determine the viability of using the assay and whether any of the aptamer species bound the target below the level associated with non-specific binding. Figure 47 depicts the first of these dot blots with a micro molar range from 20 µM HsdR immobilised to the surface through to 1 µM, as can be seen from the figure, all aptamer species were found to bind the target protein sufficiently well as to carry on reducing the concentration down to a nanomolar range.

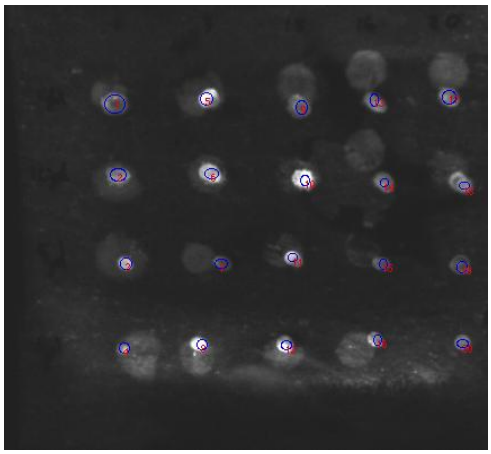


Figure 47 Dot blot HsdR binding aptamer against HsdR of decreasing concentrations. Dot blot of HBA 1, 3, 15, 16, and 20 blot from left to right against decreasing concentrations of HsdR (20 µM, 10 µM, 5 µM, 1 µM) progressing downwards, probed using streptavidin:HRP conjugate (0.125 ng/mL) and visualised using Fujifilm™ Las3500 Intelligent Dark Box.

Figure 48 is a direct repeat of the initial dot blot but, with the lower concentration range moving from 500 nM through to a no protein control. From this it can be seen that below 500 nM the amount of binding is quickly reduced to a very low level. This indicates that the

dissociation constant, where half the concentration of ligand is bound and half is free in solution lies between 1 µM and 500 nM for all the putative aptamer species. It should also be noted that the column furthest to the right contains a no-DNA control and shows a very low level response, this indicates that the streptavidin:HRP conjugate is binding specifically to the biotinylated aptamer species and can be considered a viable and high-throughput method of aptamer characterisation.

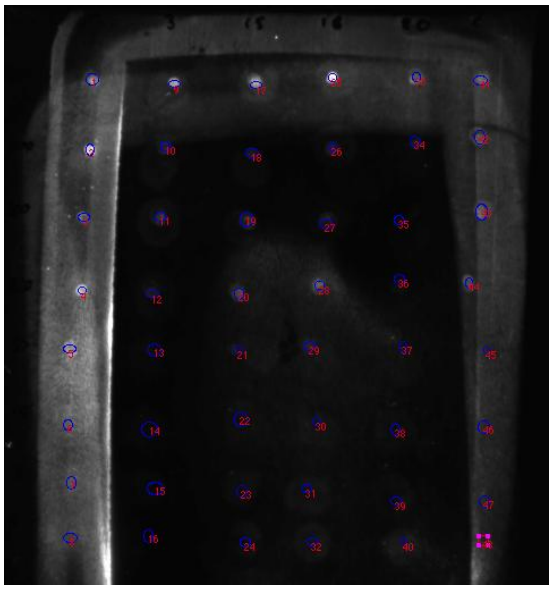


Figure 48 Repeat of HBA dot blot with lower concentrations. Repeat of HBA dot blot with lower concentrations Dot blot of HBA 1, 3, 15, 16, 20, and a no DNA control blot from left to right against decreasing concentrations of HsdR progressing downwards (500 nM, 400 nM, 300 nM, 200 nM, 100 nM, 50 nM, 10 nM, 0 nM)

4.3.5 SPR

As described before the technique of Surface Plasmon Resonance (SPR) lends itself to the verification of aptamer species that have been found to bind to a target protein. Using a 5' biotinylated aptamer that is bound to the streptavidin-dextran matrix of a pre-prepared SPR chip (Biacore®, GE Healthcare) it is possible to begin to probe the binding kinetics of the aptamer to target interactions using increasing concentrations of the antigen (in this case HsdR). The resulting data can then be used in conjunction with that of other assays to create an overall picture of the protein to ligand interaction, including dissociation constants, and aptamer to target specificity.

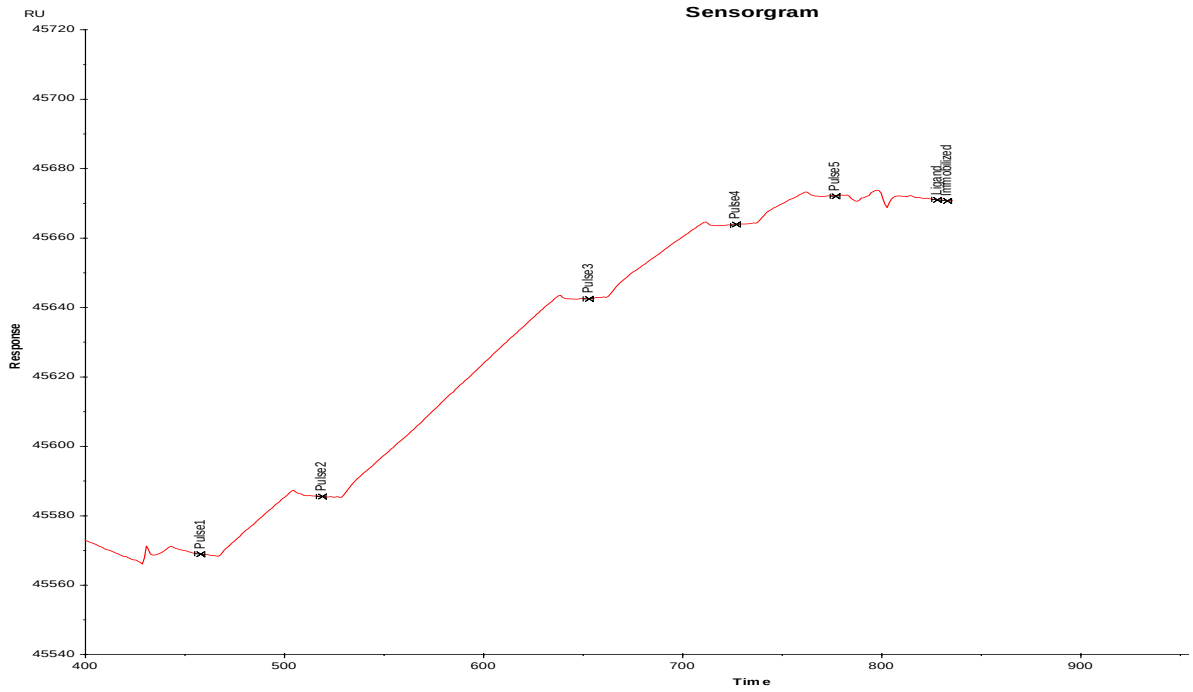


Figure 49 Surface Plasmon resonance sensorgram of the Immobilisation of HBA1. SPR sensorgram of immobilisation of 5 µM 5' biotinylated HBA applied to the pre-prepared streptavidin coated SPR chip.

Initially HsdR Binding Aptamer 1 (HBA1) was chosen for further experimentation, and once 100 RU of ligand were bound to the chip (Figure 49) representing a favourable ratio of target to analyte for signal characterisation, as recommended by the literature (BIACORE, 1998). As HsdR has a larger mass than the surface bound aptamer, 145000 Da for the HsdR-GST fusion protein compared to ~16399 Da for the 54 base pair oligonucleotide, the amount of immobilised material needed for a large shift in response units upon binding is relatively small using this instrument.

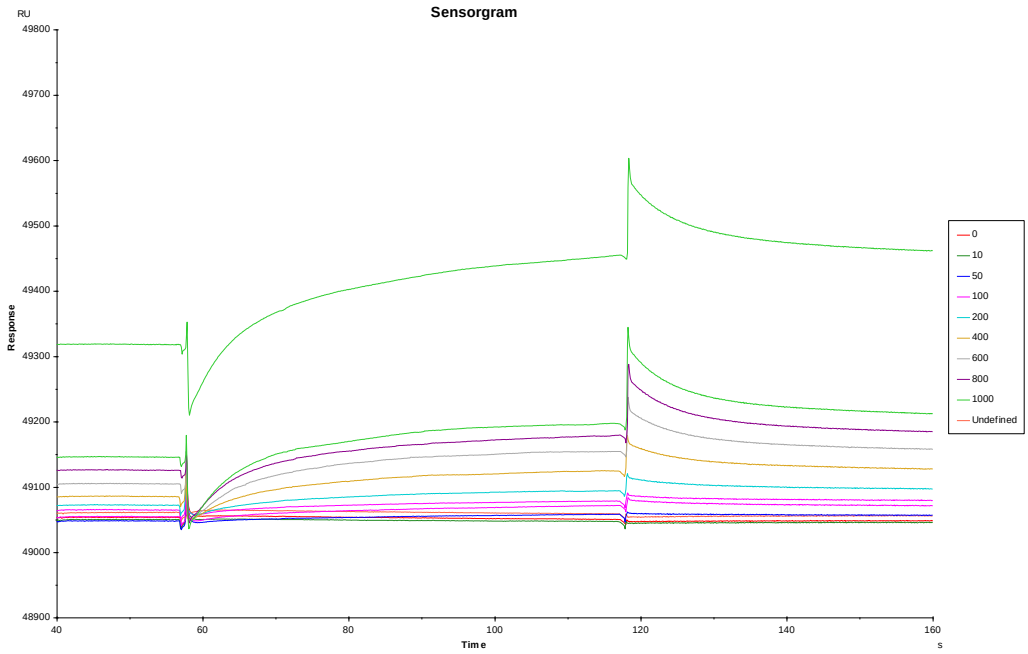


Figure 50 Sensorgram of control channel indicating increasing concentrations of HsdR attached to surface. Control sensorgram showing the binding of HsdR to the control channel, increasing concentrations of HsdR 0 nM, 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM.

As can be seen from the sensorgram (Figure 50) of HsdR binding to the control channel progressive and increasing concentrations of the target HsdR cause an increase of response units associated with persistent non-specific binding of the target. The increase of response units that builds up is not removed following the high ionic strength (2 M NaCl) regeneration events and consequently invalidates any data that can be derived from the procedure. The same can be said for the active channel that contains the immobilised HsdR binding aptamer (Figure 51) with identical levels of non-specific binding observed. An identical procedure of immobilising the candidate aptamer to the surface was carried for all the potential HsdR binding aptamers (HBA3, HBA15, HBA16, and HBA20) with the same result (data not shown).

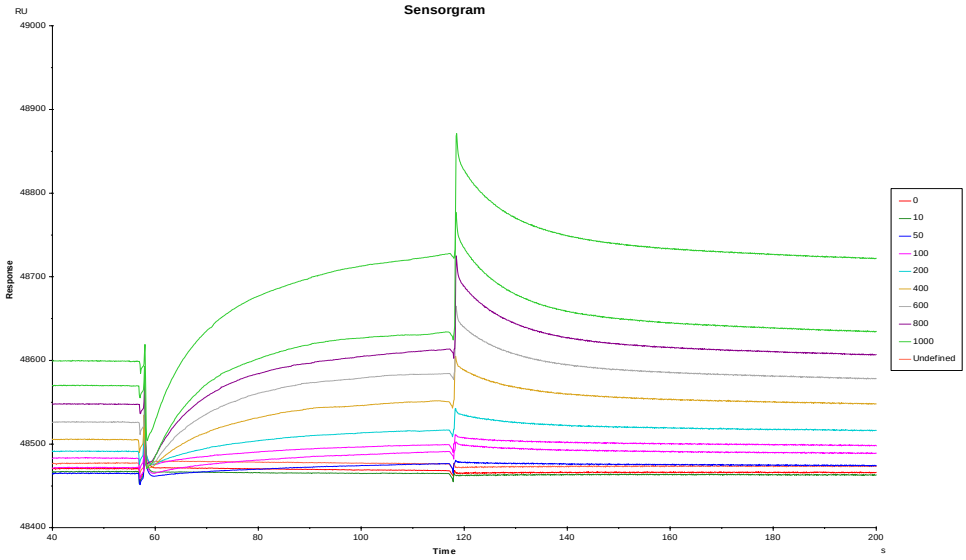


Figure 51 Sensorgram of sample channel HBA1 indicating increasing concentrations of HsdR attached to surface. Sensorgram showing the binding of HsdR to HBA1 immobilised to Biacore SA Chip, increasing concentrations of HsdR 0 nM, 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1000 nM.

4.3.6 Binding of HsdR to CM5 chip

An alternative method to the immobilisation of the candidate HsdR binding aptamers to the streptavidin coated surface of the Biacore SPR chip was sought. In order to capitalise on the affinity HsdR displays to the gold surface of the SPR sensor chip (Figure 50, Figure 51) it is possible to attach the target protein to the surface using chemistry through the primary amines found on the side chains of the polypeptide backbone of the protein. By functionalising the surface and relying on the electrostatic attraction of the HsdR to the gold sensor chip, surface immobilisation of the target can be achieved.

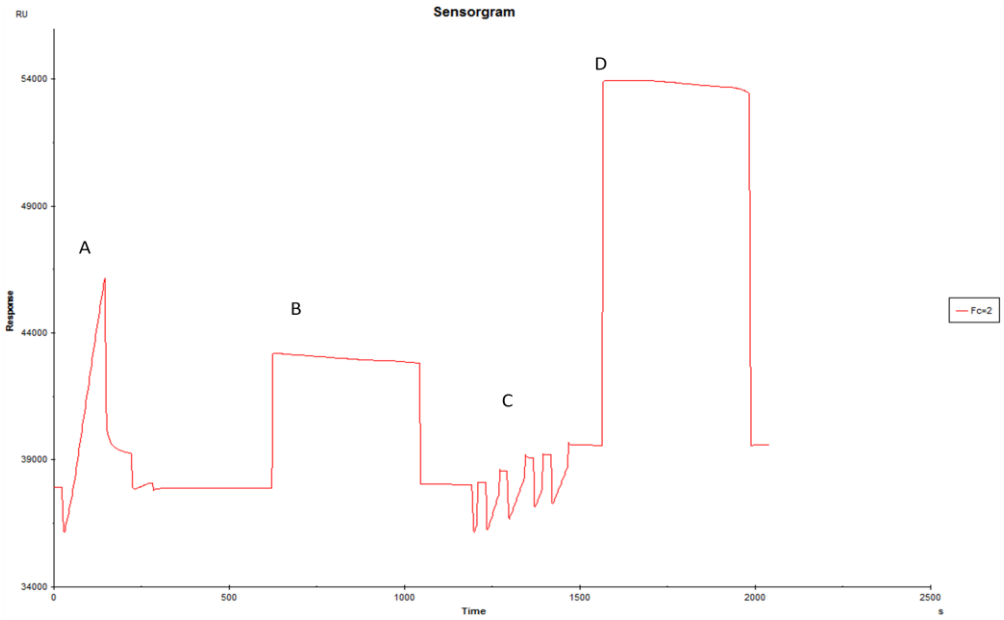


Figure 52 Immobilisation of HsdR to Biacore CM5 chip through primary amine attachment. Immobilisation of HsdR to Biacore CM5 chip through primary amine attachment. A – Concentration analysis of HsdR sample. B – Surface functionalisation using EDC/NHS. C – Successive 1 µL, 3 µL, 4 µL, 2 µL, and 4 µL pulses of 1 µM HsdR. D – 70 µL Ethanolamine wash. Increased baseline of 2075 response units bound to the surface.

Following the successful immobilisation of HsdR to the sensor chip surface (Figure 52) the candidate aptamers can be introduced to the target with any binding observable through a change in Response units. Any such change can be said to be the binding of aptamers to the target. A consequence of the reversal of the immobilisation of the target rather than the aptamer is the relative change in response units associated with a binding event. HsdR can be said to be a large protein (~145 kDa native molecular weight) which is a much larger mass than that associated with the aptamers (8582.6 Da for a 28 base pair aptamer). Due to the large relative mass ratio (1:16 HsdR aptamer) the detection of aptamers upon binding is difficult using SPR methodologies, it is not however, impossible.

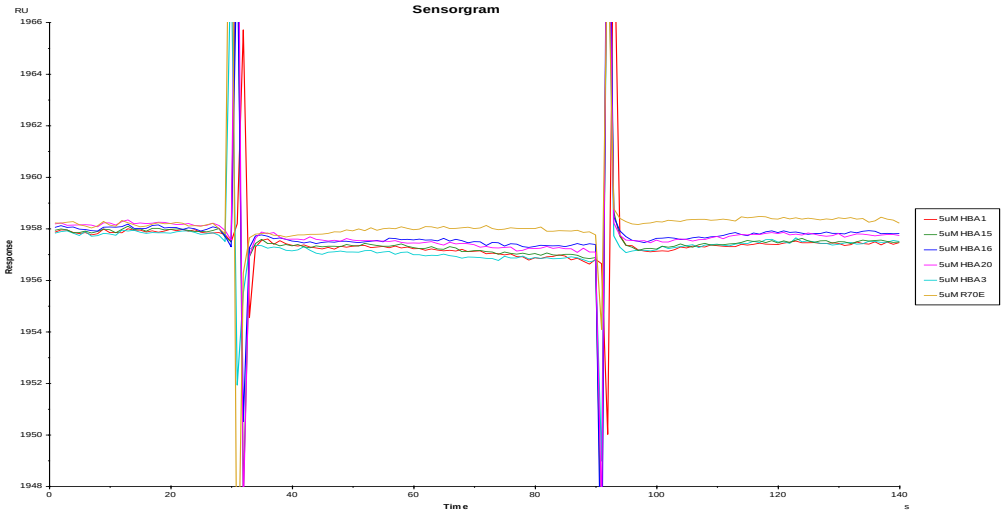


Figure 53 SPR analysis of HsdR binding aptamers binding to immobilised HsdR. SPR analysis of HsdR binding aptamers binding to immobilised HsdR. Surface bound HsdR (Figure 52) introduced to candidate aptamers according to legend. 5 µM HBA1 , 5 µM HBA3, 5 µM HBA15, 5 µM HBA16, 5 µM HBA20, and 5 µM control DNA TBA R70E.

As can be seen from the sensorgram Figure 53, there is no significant change in response unit observed for any of the candidate aptamers, this lack of binding can be attributed to the protein being in a non-native state that is the result of the immobilisation method, it may also be attributed to the candidate aptamers not forming the secondary structure motifs required for binding in the analysis buffer.

Unfortunately the issue of non-specific binding could not be solved, it being neither the GST tag, nor the buffer in which the HsdR was in. After the series of higher ionic strength buffers and increasing and varied use of detergents to discourage HsdR interaction with the control channel as well as the active channel the reliable use of HsdR with this method of SPR proved redundant. The persistent non-specific binding of the protein to the unprepared control channel could be due to electrostatic attraction of HsdR to the gold surface, although this is not investigated here. Also, due to the comparatively expensive nature of reversing the experiment around, with HsdR bound to the chip and the aptamer species flowed over, and with the difficulty of immobilising native and folded HsdR to a surface through primary amine coupling an alternative method of assaying the potential aptamer species was sought.

Table 8 Table of potential aptamers and estimated dissociation constants

Aptamer name	Apparent dissociation constant (K_d)
HBA1	200 nM
HBA3	450 nM
HBA15	450 nM
HBA16	450 nM
HBA20	650 nM

4.3.7 Activity assay

A previously reported method for assaying the restriction activity of the HsdR subunit could be used to further characterise the HsdR binding aptamers. By establishing whether the aptamer, upon binding, interferes with the known level of cleavage activity associated with the HsdR subunit a direct comparison that can give insight into whether an aptamer is directly binding one of the active epitopes of the protein.

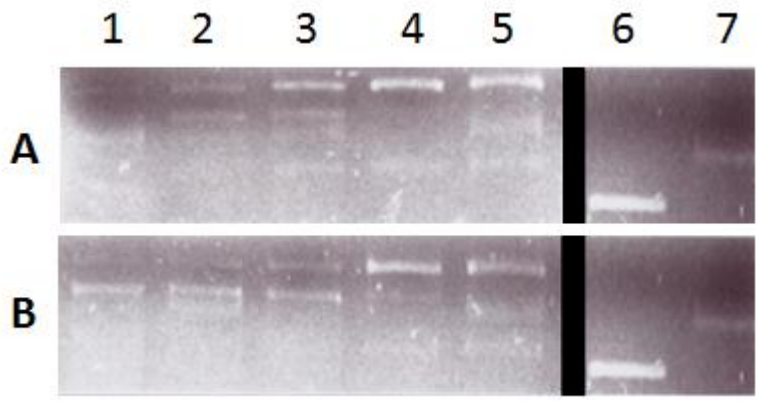


Figure 54 Initial EcoR124I digestion of pCFD30. Restriction Inhibition Assay. Lane 1 HsdM:HsdR ratio 0, Lane 2 HsdM:HsdR ratio 1:1, Lane 3 HsdM:HsdR ratio 1:2, Lane 4 HsdM:HsdR ratio 1:5, Lane 5 HsdM:HsdR ratio 1:10, Lane 6 No Enzyme Control, Lane 7 EcoR1 restriction control. A. No Aptamer control B. Restriction Assay with 10 uM anti-ATP aptamer.

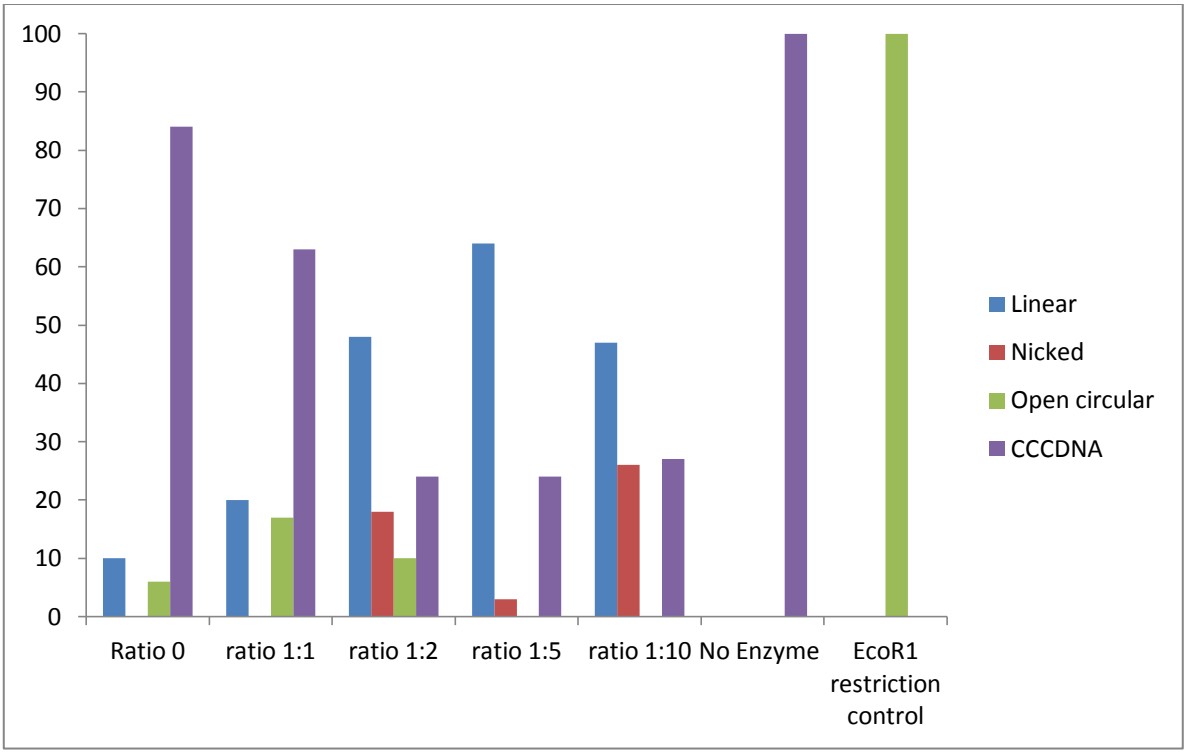


Figure 55 Control Restriction Assay band analysis. Control Restriction Assay band analysis of EcoR124I digestion of pCFD30. Image analysed with ImageJ software and band intensity quantified and expressed as a percentage of total response.

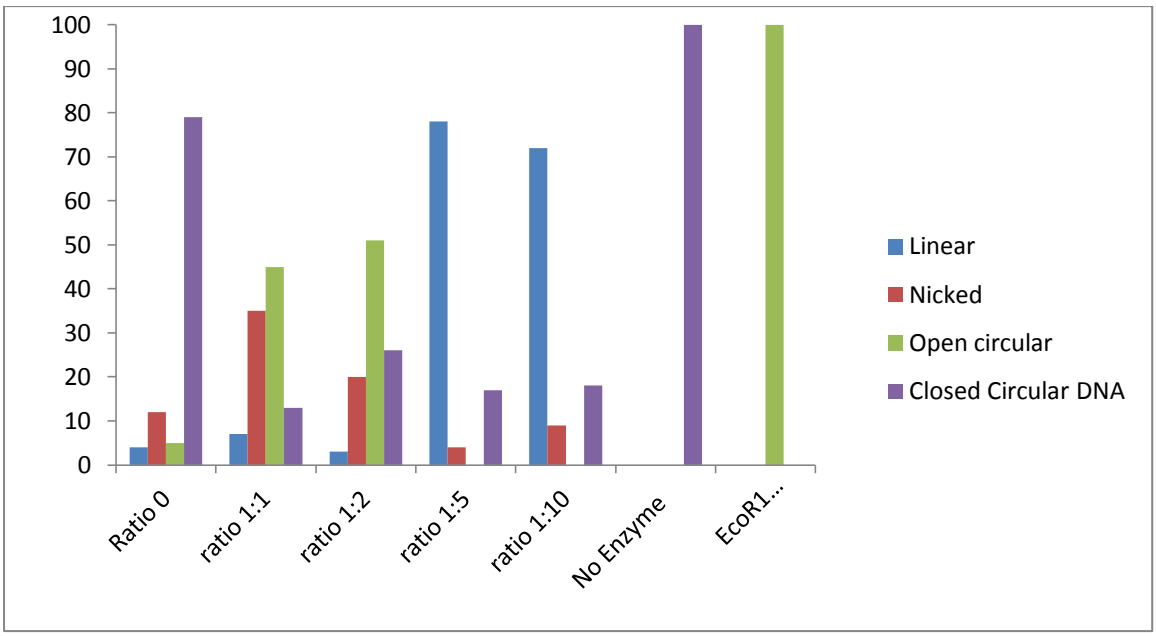


Figure 56 Anti-ATP Aptamer Restriction Assay band analysis. Anti-ATP Aptamer Restriction Assay band analysis A repeat of experiment Figure 55 with 10 µM DNA anti-ATP aptamer added to the reaction. Image analysed with ImageJ software and band intensity quantified and expressed as a percentage of total response.

Analysis of the relative intensities of the digested bands (Figure 55) indicates that the level of restriction of the pCFD30 substrate from supercoiled linear is inhibited by the presence of

the DNA ATP aptamer. Using the data generated it can be seen that at the 1:2 ratio of HsdM:HsdR approximately 48-50% of the substrate is digested after 2 hours, and from the analysis of the inhibition assay with the added DNA ATP aptamer (Figure 56) the restriction is inhibited to 4% of the total DNA present. The level of restriction inhibition observed can be used as proof of concept for the validation of the restriction assay as a method for probing possible aptamer binding sites.

4.4 SELEX to isolate Human Rhinovirus 14 3C binding aptamers

After the success of isolating a series of aptamers to the fusion protein HsdR, a distinct but relatively similar protein target was sought to validate the technique of affinity SELEX as realised in this investigation. The protease Human Rhinovirus 14 3C (HRV143C) is commercially available as a fusion protease known as HRV143C(GE Healthcare®). Like GST-HsdR, HRV143C is purified using a glutathione-S-Transferase fusion protein partner the bindings reversibly with glutathione that is bound to an affinity matrix. In an identical method to that described for HsdR SELEX, HRV143C SELEX relies on the affinity of the GST fusion tag to immobilise the target to the affinity column. With progressive iterations of the SELEX screening and amplification process, a final aptamer pool was cloned into the vector pGEM-T-Easy™ (Promega) after 13 rounds. 20 candidate clones were sequenced with a total of 94 potential aptamer sequences recovered (Table 3).

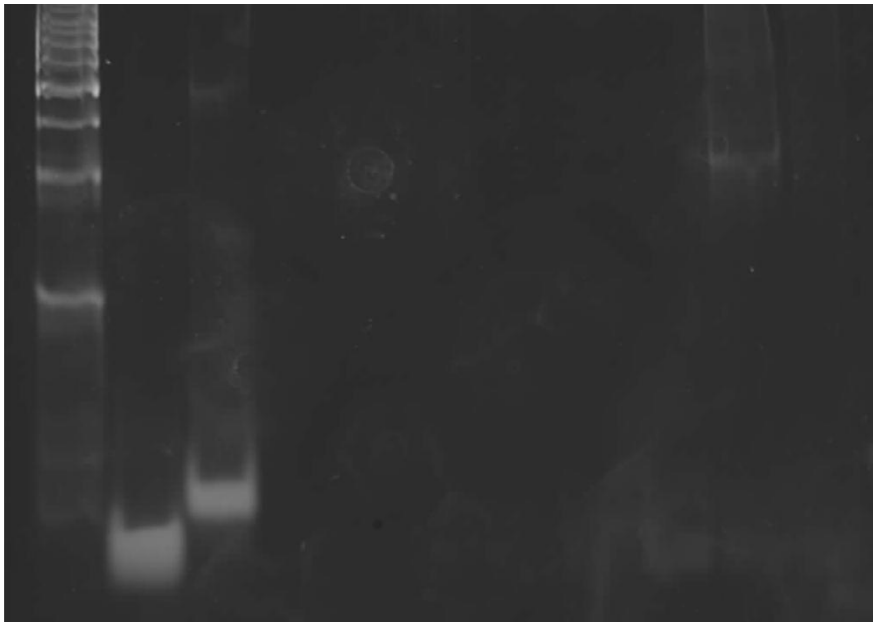


Figure 57 **6% Native acrylamide gel of rounds of SELEX against protein target HRV143C. 15 % Native acrylamide gel showing the immobilisation of PBA aptamer library. Lane 1: Hyperladder V, Lane 2: SELEX Forward Primer, Lane 3: SELEX reverse Primer, Lanes 4 – 10: HRV143C SELEX rounds 4 – 10 respectively. Stained with SYBR™ (Promega)**

Table 9 Multiple sequence alignment of Recovered Sequences for HRV143C binding aptamers

	Aptamer sequence		
1	-------CCCACACACTACTCTGCCGATTTGGT-----	19	-----CCCATCGACAGTCTG-CTTCAGATCGGT-----
14	-------CCCACACACTACTCTGCCGATTTGGT-----	39	-----CCCATCGACAGTCTG-CTTCAGATCGGT-----
17	-------CCCACACACTACTCTGCCGATTTGGT-----	37	----CCTCATCAGTAGTCAT-CATCGT----------
25	-------CCCACACACTACTCTGCCGATTTGGT-----	6	----CCCAAT-GAACATGCCACAGTTGGTCGT------
70	-------CCCACACACTACTCTGCCGATTTGGT-----	13	----CCCAAT-GAACATGCCACAGTTGGTCGT------
75	-------CCCACACACTACTCTGCCGATTTGGT-----	22	----CCCAAT-GAACATGCCACAGTTGGTCGT------
58	-------CCCACACACTACTCTGCCGATTTGGT-----	48	----CCCAAT-GAACATGCCACAGTTGGTCGT------
68	-------CCCACACACTACTCTGCCGATTTGGT-----	7	----CCCAAT-GAACATGCCACANTTGGTCGT------
56	-------CCCACACACTACTCTGCCGATTTGGT-----	42	----CCCAAT-GAACATGCCANANTTGGTCNT------
57	-------CCCACACACTACTCTGCCGATTTGGT-----	31	----CCCAAT-GAANATGCCANANTTGGTCNT------
38	-------CCCACACACTACTCTGCCGATTTGGT-----	32	----CCNANCAAAAGGTGCNATGCTTTACN--------
50	-------CCCACACACTACTCTGCCGATTTGGT-----	43	----CCNANCANAANGTGCNATGCTTTACNGN----- -
16	---CCAAGCGGGATAC-ACACTACTGGGACGC------	23	----CCGAGCAGAAGGTGCGATGCTTTACGGT------
24	---CCAAGCGGGATAC-ACACTACTGGGACGC------	53	--------CCAAGCGGGCGCGAGAACAACGTACGGT- -
63	---CCAAGCGNNATAC-ACACTACTGANANNC------	66	--------CCAANCGGGCGCNAGAACAACGTACGGT- -
18	-----CCTCGGGACGGTGCTTCACCCTGCTGGT-----	5	----------AGGCGGGGGGACTGACAATGTG------
26	-----CCTCGGGACGGTGCTTCACCCTGCTGGT-----	62	--CCGGGGCACACTGACGGGACTAACAGCGCGC-- --
2	---CCTCGGGACGGTGCTTCACCCTGCTGGT-----	67	--CCGGGGCACACTGACGGGACTAACAGC---------
20	-----CCCACAGACATTAGGACGCTACATGGGGT----	60	-CCCGGGAATAACCGTACGAGTTCGGGGT---------
21	-----CCCACAGACATTAGGACGCTACATGGGGT----	11	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------
55	-----CCCACAGACATTAGGACGCTACATGGGGT----	12	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------

59	-----CCCACAGACATTAGGACGCTACATGGGGT----	27	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------
28	-----CCCACAGACATTAGGACGCTACATGGGGT----	49	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------
3	-----CCCACAGACATTAGGACGCTACATGGGGT----	51	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------
76	-----CCCACAGACATTAGGACGCTACATGGGGT----	74	---CCCGGGCGGACAGCAGGCTGTTCGTTGC-------
45	-----CCCACAGACATTAGGACGCTACATGGGGT----	15	---CCCGGGCGGACAGCAGGCTGTTCGTTG--------
10	-----CCCACAGACATTAGGACGCTACATGGGGT----	47	-----CCGGCGGGCGGGAGGCATCTGAGGGAGT----
52	-----CCCACAGACATTAGGACGCTACATGGGGT----	33	---------CCAACCGGAATCCAGTCGAGGT-------
36	-----CCCACAGACATTAGGACGCTACATGGGGT----	4	--------CCACATTGTCCTTCCTTGTGTGT-------
46	-----CCCACAGACATTAGGACGCTACATGGGGT----	8	--------CCACATTGTCCTTCCTTGTGTGT-------
40	-----CCCACAGACATTAGGACGCTACATGGGGT----	44	------TCGTGCGTAACGTGTGCTTGGTGGT-------
69	-----CCCACAGACATTAGGACGCTACATGGGGT----	65	---CCATCGT-CGTNAATTGTCTTCNGTGGT-------
72	-----CCCACAGACATTAGGACGCTACATGGGGT----	54	CCCATGACCTGCGTTCATCG-TGTTCGGT---------
73	-----CCCACAGACATTAGGACGCTACATGGGGT----	61	--CCGGCTGTGCTTTTACTGGTGTTGGGT---------
30	-----CCCACAGACATTANGACGCTACATGGGGT----	29	--CGTTTTCTTGATGTTTTTGCATTGGT----------
41	-----CCCACANACATTANGACGCTACATGGGGT----	9	-------CATGGCAGGG--TGCANTTGTGGT-------
71	-----CCCACAGACATTAGGACGCTGCATGGGGT----	35	----CTCTATGGCAAGCGTTGTGTTTGTGGT-------
	64 -----		
	CCCACATACATTAGGACNCTACATGGGGTGCGC		

A multiple sequence alignment highlighted the fact that recurring sequences of CCCACACACTACTCTGCCGATTTGGT and CCCACAGACATTAGGACGCTACATGGGGT were common. The ten most common recurring sequences were chosen for further investigation using the methods refined from the HsdR binding aptamers.

Table 4. Table of PBA candidates, along with sequence and predicted dissociation constants determined by dot blot

Aptamer name	Aptamer Sequence	Predicted dissociation constant (K_d)
PBA 1	CCCACACACTACTCTGCCGATTTGGT	~900 nM
PBA 2	CCAAGCGGGATACACACTACTGGGACGC	~7.5 µM
PBA 3	CCCACAGACATTAGGACGCTACATGGGGT	~950 nM
PBA 4	CCCATCGACAGTCTGCTTCAGATCGGT	~1.25 µM
PBA 5	CCCAATGAACATGCCACAGTTGGTCGT	Nil
PBA 6	CCGGGGCACACTGACGGGACTAACAGC	Nil
PBA 7	CCCGGGCGGACAGCAGGCTGTTCGTTGC	~950 nM
PBA 8	CCGGCTGTGCTTTTACTGGTGTTGGGT	~7.5 µM
PBA 9	CGTTTTCTTGATGTTTTTGCATTGGT	~5 µM
PBA 10	CTCTATGGCAAGCGTTGTGTTTGTGGT	~5 µM

4.4.1 Non-denaturing HPLC of HRV143C SELEX rounds 1-9

HPLC analysis of the progressive rounds (Figure 58) that resulted in the enhanced HRV143C aptamer pool proved largely inconclusive. A slight refinement of the reduced peak with a retention time of 3 minutes is observable, although greatly reduced from the first round (in blue) the peak can be seen to diverge from one large peak to two combined peaks in rounds 3 to 9 of HRV143C SELEX. Although the SELEX process can be deemed to be successful (Figure 57), in the case of the HRV143C SELEX process, the distinction between the aptamer libraries cannot be said to be sufficiently different as the HsdR binding aptamer according to the HPLC analysis (Figure 44). This may be due to the structural variation of the enriched HRV143C SELEX libraries not being as distinct as those of HBA SELEX.

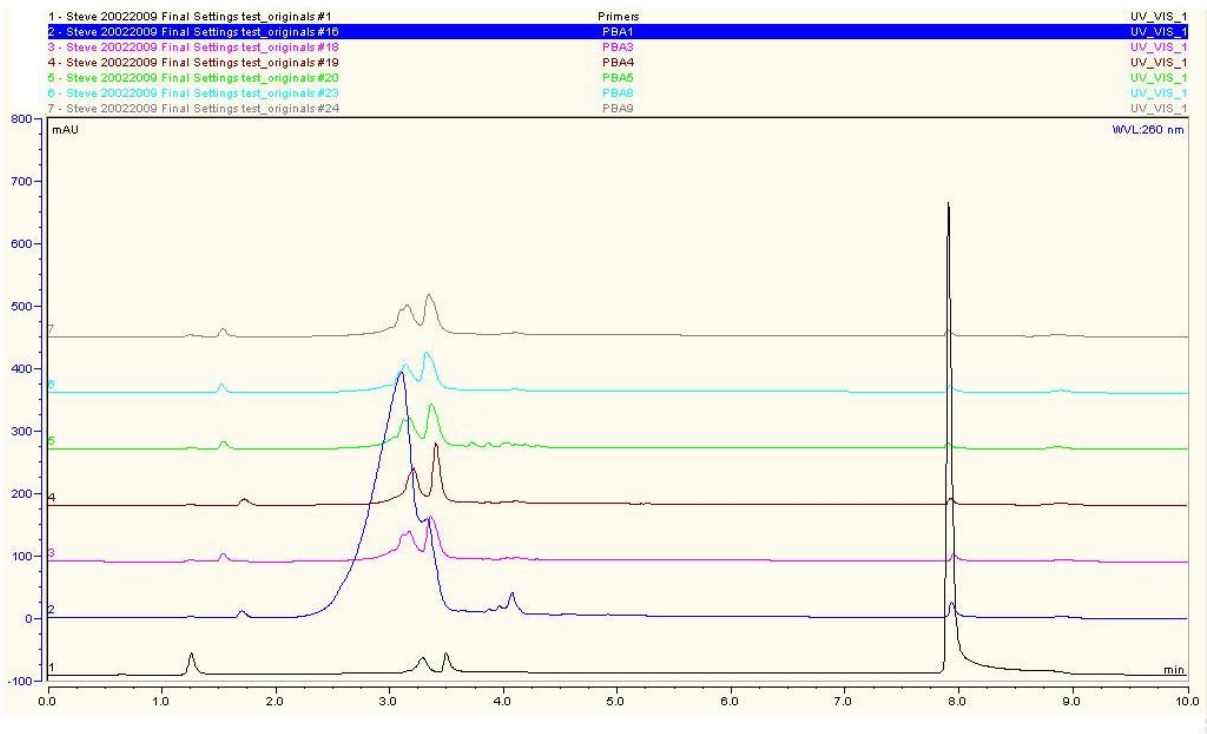


Figure 58 Non-denaturing HPLC of HRV143C SELEX rounds 1-9. Chromatograms of Progressive rounds of HRV143C SELEX analysed using the Dionex 3000 HPLC system and DNAPac column. The peaks symbolize the absorption of UV light at λ260 nm and represent the refinement of the DNA aptamer library through rounds 1,3,4,5,8 and 9 of HRV143C SELEX.

4.4.2 Dot Blot

Following the established methodology for the rapid assay and validation of a potential aptamer pool described above for the HsdR binding aptamer the candidate sequences of the HRV143C binding aptamer (PBA). Each sequence carries a 5' biotin attachment for

detection using the streptavidin HRP conjugate, and was incubated with a series of concentrations of HRV143Cthat had been dried onto a nitrocellulose membrane and blocked with 3% Bovine Serum Albumin. As a result it is possible to detect the concentration at which the amount of binding falls from 100%, an extrapolation from this is that 50% of K_{max} is K_a/K_d or K_d which can give an estimate of the dissociation constant of each particular species of aptamer in the specified conditions. $[A]_{Total}$

$$\frac{[AB]}{[\mathrm{A}]\mathrm{Total}}$$

This insight can be used to contribute to the decision as to which of the PBAs to take forward for further, more concentrated, investigation methods, such as SPR or ultimately structural modelling methodologies (protein crystallography, and Nuclear Magnetic Resonance studies).

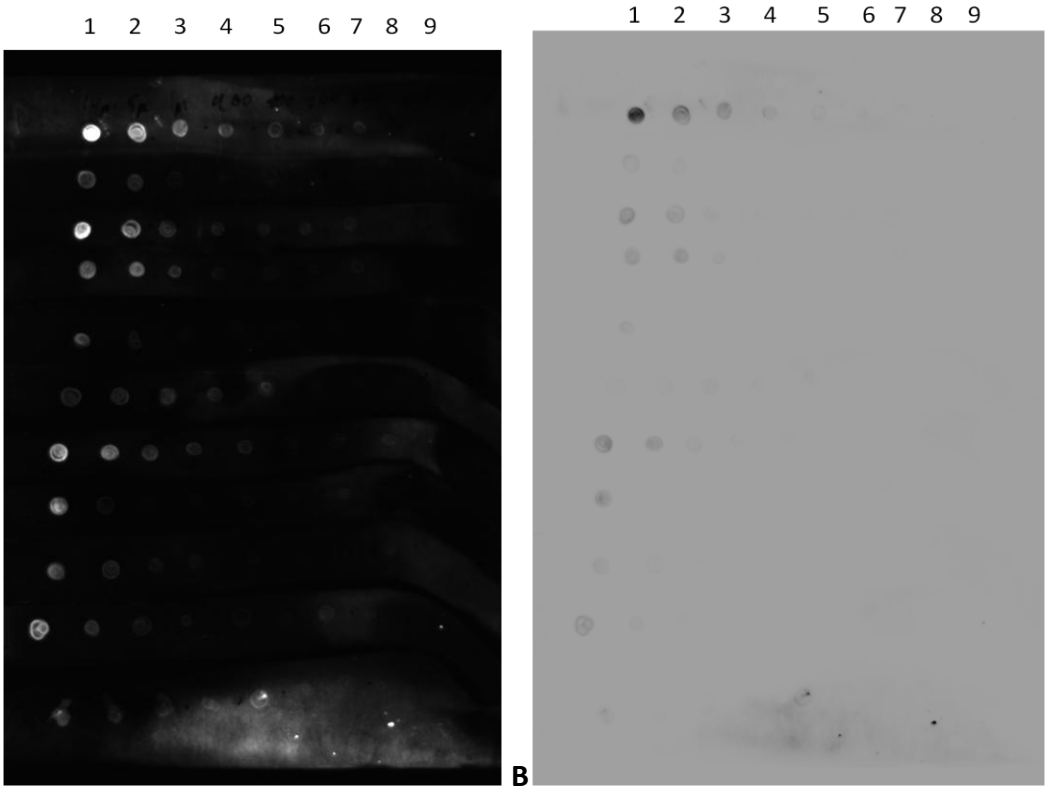


Figure 59 Dot blot assay of HRV143C Binding Aptamer (PBA) species 1-10 against decreasing concentrations of HRV143C. Dot blot of 5 µM PBA 1 through to 20 at a concentration of from top to bottom against decreasing concentrations of HRV143C (10 µM, 5 µM, 1 µM, 900 nM, 800 nM, 700 nM, 600 nM, 500 nM) progressing from left to right, probed using streptavidin:HRP conjugate (0.125 ng/mL) and visualised using Fujifilm™ Las3500 Intelligent Dark Box. Row 11 is the no DNA control. A – Native dot blot, B-Inverted Pixel repeat

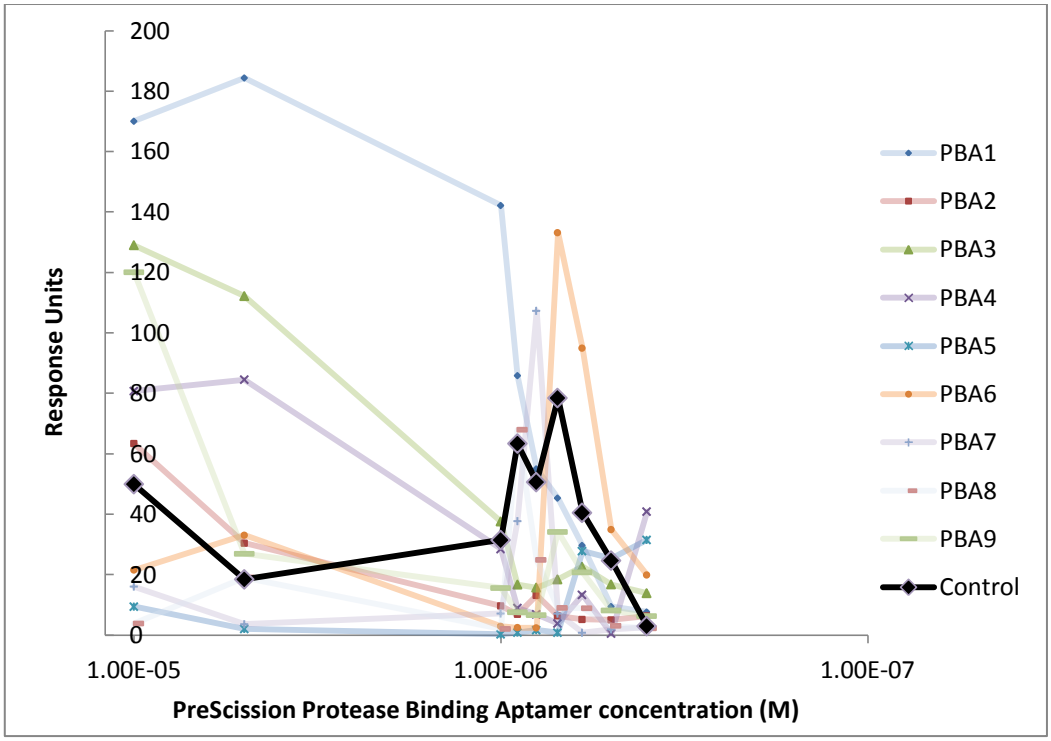


Figure 60 Summary of PBA dot blots Chemiluminescent responses. Summary of PBA dot . Graphic representation of results of the dot blots (Figure 59). Data generated by intensity of response divided by area in ImageJ .

From the dot blot assays (Figure 59) and the subsequent values resulting from the analysis (Figure 60) the aptamer sequences that have been seen to have a response that indicates aptamer binding can also have a dissociation constant predicted, although this prediction has a series of caveats for the reliability of the data produced from this method as mentioned above, the predictions are detailed in the table below (Table 10)

Table 10Table of PBA candidates, along with sequence and predicted dissociation constants determined by dot blot

Aptamer name	Aptamer Sequence	Predicted dissociation constant (K_d)
PBA 1	CCCACACACTACTCTGCCGATTTGGT	~900 nM
PBA 2	CCAAGCGGGATACACACTACTGGGACGC	~7.5 µM
PBA 3	CCCACAGACATTAGGACGCTACATGGGGT	~950 nM
PBA 4	CCCATCGACAGTCTGCTTCAGATCGGT	~1.25 µM
PBA 5	CCCAATGAACATGCCACAGTTGGTCGT	nil
PBA 6	CCGGGGCACACTGACGGGACTAACAGC	nil
PBA 7	CCCGGGCGGACAGCAGGCTGTTCGTTGC	~950 nM

PBA 8	CCGGCTGTGCTTTTACTGGTGTTGGGT	~7.5 µM
PBA 9	CGTTTTCTTGATGTTTTTGCATTGGT	~5 µM
PBA 10	CTCTATGGCAAGCGTTGTGTTTGTGGT	~5 µM

4.4.3 Activity Assay

The simplistic method in which candidate aptamer pools are screened and amplified and ultimately arrived at, largely leaves a gap in the knowledge of the location or epitope to which each individual aptamer is preferentially binding to. In an effort to decipher the location of the primary binding epitope of each individual candidate PBA, an assay was required that utilises the primary function of HRV 14 3C protease. The fusion protein clone of EcorR124I HsdR subunit used for the expression of GST-HRV143C-HsdR contains a linker region that holds the peptide recognition sequence and subsequential cleavage site for HRV 14 3C . By creating the conditions in which HRV 14 3C will cleave its peptide substrate, in this case the glutathione-S-Transferase subunit of the larger fusion protein, it is possible to gain an insight into whether any of the proposed aptamer sequences occupy the active site of HRV 14 3C. Through comparison to the known cleavage event, any reduction in overall cleavage can indicate inhibition through competition. The resultant gel, Figure 61, gives a clear result in that a total lack of inhibition can be observed. Through comparison to the control in lane 12, it is possible to see that the cleaved GST tag will migrates at ~24000 Da in a denaturing SDS page gel. HRV 14 3C migrates at ~56000 Da with the completely cleaved HsdR subunit migrating through the gel at ~121000 Da, which is to be expected as the native fusion protein is known to be 145000 Da from previous work.

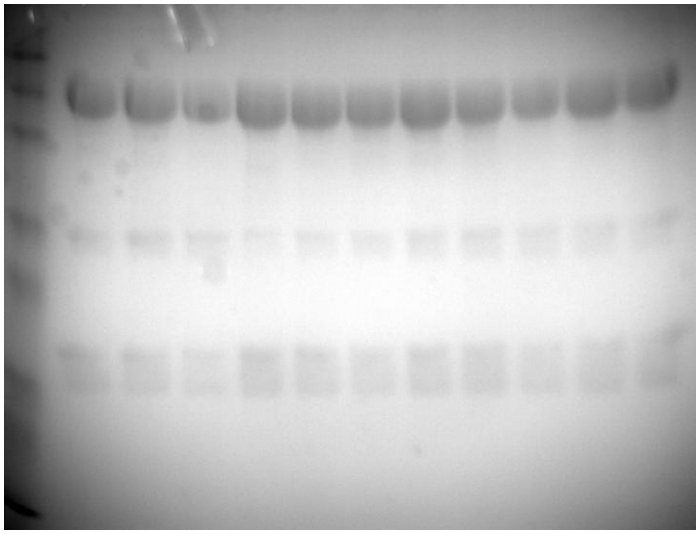


Figure 61 HRV143C Cleavage of HsdR . 10% SDS PAGE gel of 100 ng HsdR incubated with 2 NIH units of HRV143C (2 mg/ml) and 10 µM PBA, for 4 hours at 24°C. Lane 1 Benchmark Prestained protein ladder (Promega), Lanes 2-11 PBA 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, Lane 12 No aptamer control.

Using assays such as the inhibition of cleavage assay and the dot blot, Figure 61, and Figure 59 respectively, a larger picture for where an aptamer is binding can be drawn. Although the techniques are limited, without the use of other, further studies, primarily focused on the structural properties of the protein and their aptameric ligands, it is difficult to construct the exact nature of the interaction. This is not the purpose of this investigation, although there continues to be an interest in the further examination of the PBA:HRV143C such as a cure for the common cold (as HRV143C is the virus responsible for this) and much further work can be undertaken in order to further characterise the interaction.

5 Production of novel aptamers- Separation methods

5.1 The Methylase subunit of EcoR124I

The methods presented in this chapter are designed to further highlight the ease with which SELEX can be achieved, and the variation of proteins and targets to which aptamers can be isolated. Through the use of gel electrophoresis, HPLC, and nanopore molecule sorting, aptamer library refinement can be achieved, highlighting the ease of which aptamers can be raised to targets. This chapter will also demonstrate the enrichment of random DNA libraries towards a small molecule target. This will in effect complete the desired suite of aptamer derivation techniques in a typical laboratory environment.

The methylase, (MTase) subunit of EcoR124I is a 58kDa (Taylor *et al.*, 1994) protein that associates with one HsdS specification subunits, to form the modification portion of the enzyme, and with two HsdR subunits and to form the active restriction modification system (Janscák *et al.*, 1996). The subunit is of interest because of an extensive characterisation that has progressed over a period of 30 years and in conjunction with the HsdR subunit combine to form the restriction modification system EcoR124I in the configuration $R_2M_2S_1$ (Janscák *et al.*, 1998).

Given the ability of the MTase subunit to specifically bind double stranded DNA at the known recognition sequence it is an interesting point to note whether the ssDNA aptamers derived through the SELEX process will bear any resemblance to the published DNA recognition sequence of EcoR124I (Yuan *et al.*, 1980) (Studier & Bandyopadhyay, 1988, Dryden *et al.*, 1997, Szczelkun *et al.*, 1997). Aptamers have been isolated to DNA binding proteins previously, Bai *et al.* (Bai *et al.*, 2005) islotated ssDNA aptamers to the DNA binding protein Rv3676 with the resulting sequence bearing a large resemblance to the known recognition sequence. The outcome of this could point to some enticing conclusions. For example, if the sequence does resemble the dsDNA binding site, are the aptamers forming a novel secondary structure, or merely mimicking the sequence found in nature? The outcomes of this may alter the understanding of the methodology of SELEX, and the perceived ability of the technique to isolate novel binding sequences. Conversely, it may also point to the diversity of ssDNA structural motifs and an ability of ssDNA to form the

structure(s) required to arrange the required bases in correct configuration for recognition through base stacking, stem loops, and guanine quadraplexes ,for example.

5.2 Nitrotyrosine

Nitrotyrosine (NT) is a modified amino acid that can act as a biomarker for a number of medical conditions, such as diabetes (Ceriello *et al.* 2002), and in disease affected tissues (Bruijn *et al.*, 1997). Nitrotyrosine is commonoly used as a marker molecule as it absorbs light at λ=450 nm (Walker, 1996), and it is because of this property that the molecule has been selected to be the target of this small molecule selection.

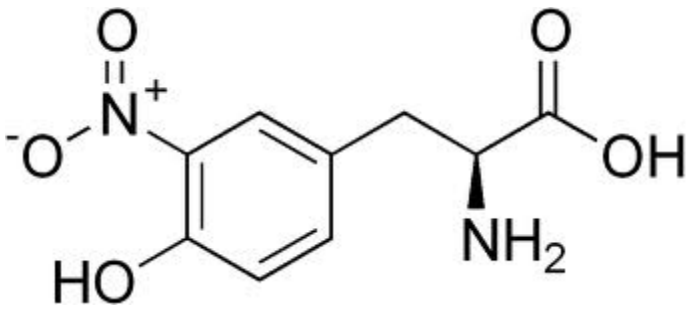


Figure 62 Chemical Structure of 3-Nitrotyrosine. Cartoon Depiction of the chemical structure of Nitrotyrosine.

5.3 Gel Electrophoresis SELEX (GELEX)

This novel method lends itself to the derivation of aptamers to previously characterised proteins, unknown proteins, or even complex mixtures of multiple proteins providing there is sufficient difference in charge and shape of the constituents. This method is similar to the published methods CE-SELEX, (Mosing and Bowser 2009) and NECEEM (Berezovski & Krylov 2002), but varies in the bulk nature of the method. The Capillary Electrophoresis methods, both work in minute quantities, and this will save much on sample preparation. But, if the amount of protein is not an issue, or it is more amenable to produce large amounts of protein rather than purchase an expensive piece of equipment, GELEX may provide an alternative.

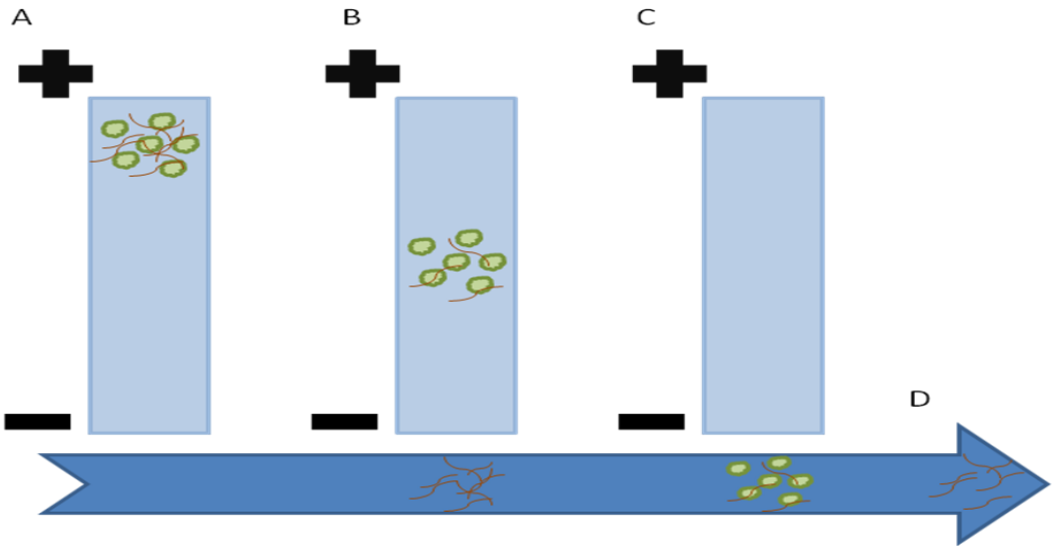


Figure 63 Schematic of GELEX process. Schematic of GELEX process, A. Aptamer + target mixture B. Current applied and bound and unbound species separate C. Distinct fractions collected with bound species going on for further amplificiation and the next round of selection.D. Unbound species removed

Using this method releases the target from the necessity of immobilisation, this in turn will give rise to aptamers that have been raised to native protein in the pH and ionic conditions determined to be the most beneficial for activity of the protein or for downstream applications of the required aptamer such as within a biosensor. The persistent drawback of the technique remains the length of time required to separate the bound and unbound libraries from each other.

5.3.1 Isolation of Mtase binding aptamers using GELEX

The GELEX process begun was incubation of the purified MTase subunit of the EcoR124I restriction endonuclease with an aptamer library of ~10^{13} species. The mixture was separated using a 20mm 6% Native acylamide gel and subjected to a 100v current for 4 hours at 4^{o}C and 64 1 mL fractions collected. All fractions found to be containing protein (Figure 69) were pooled and amplified, the process is repeated for 3 rounds, as recommended by Bowser (REF) with the final library cloned using the pGem-T-Easy vector system (Promega), and finally sent away to be sequenced (Cogenics UK).

The BioRad Miniprep Cell (BioRad Ltd, US) is a device that has been designed to be used for the separation of proteins in native and denaturing environments. The method presented here is an alteration from the intended use, but, it is a display of the versatility of the SELEX method.

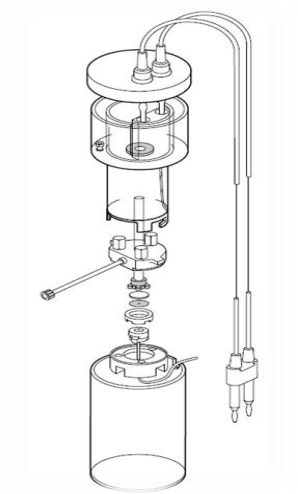


Figure 64 Cartoon of the Biorad Mini-prep Cell. The Biorad Mini-prep cell is a tool intended for the separation of proteins in bulk according to size using a native agarose gel system.

Using the Bio-Rad Mini-prep Cell (Figure 64), buffer is flowed across the bottom of an acrylamide gel and fractionated. Molecules are sorted by electrophoretic separation because their intrinsic overall net charge and the bound and unbound portions are partitioned in this manner (Figure 63). The fraction containing bound species of library and target are sufficiently separated from the un-bound and the resulting fractions are pooled and amplified. This procedure can then be repeated until a suitable library of potential aptamers is elucidated. This process has been termed GELEX.

The GELEX process was undertaken using the Mtase subunit as a model protein. Although the potential issue with raising aptamers to a DNA protein have been mentioned above, the site is known to be specific to dsDNA whilst the aptamer starting library is ssDNA. Also later analysis can identify any sequences that do contain the recognition sequences and they will be discarded. Additionally during the partitioning process the DNA occupying the recognition site may be out-competed by a higher affinity ligand such as a candidate DNA aptamer. Through multiple rounds of GELEX these ligand with a higher affinity for the will be preferentially selected for, before becoming the dominant species for that epitope. Finally, the equilibirum will shift towards the higher affinity ligands and the lower affinity ligand will be washed away in the separation portion of the process **B** (Figure 63).

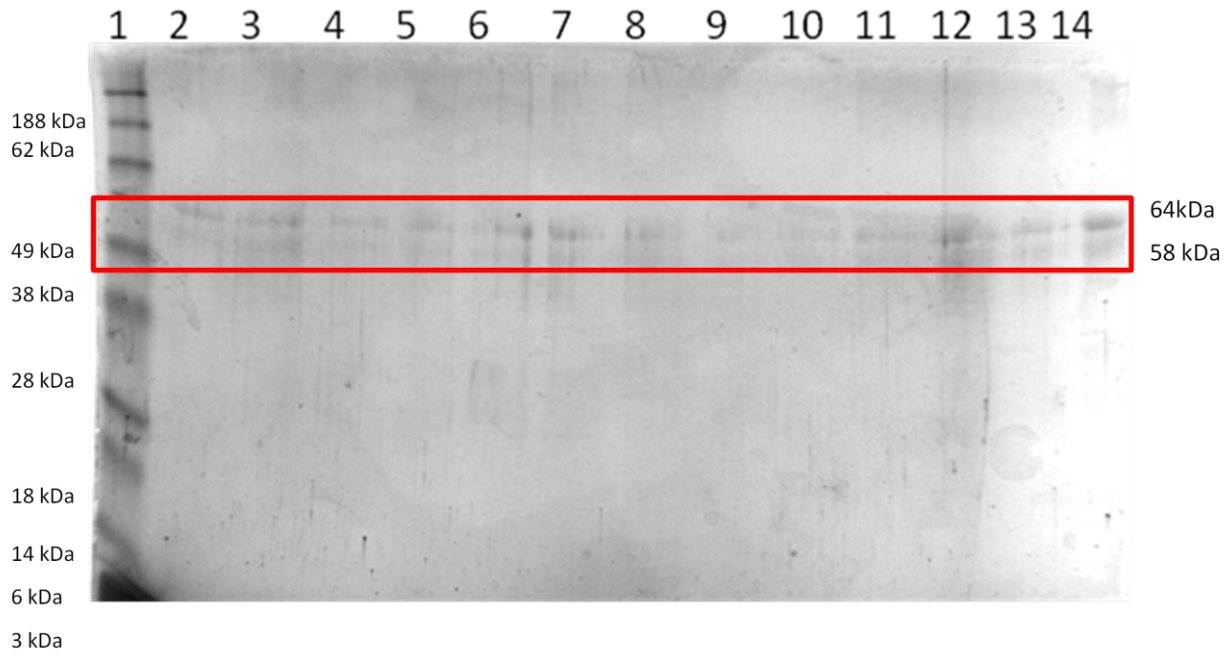


Figure 65 Recovery of HsdM and HsdS domains of EcoR124I during GELEX. 10% SDS acrylamide gel Coomassie stained depicting the recovery of HsdM (58 kDa) and HsdS (64 kDa) subunits of the EcoR124I R-M system from the BioRad Prep cell. Each fraction was to subjected to PCR amplification then pooled prior to base denaturation in preparation for the next round of screening. Lane 1 is SeeBlue pre-stained protein ladder, lanes 2-13 are samples of fractions 50-62 of GELEX

Figure 65 is a SDS PAGE gel depicts the bands of protein found across 12 fractions that result from the GELEX process. The fractions were pooled and the protein removed using phenol chloroform (Materials and Methods 5.2) extraction before the DNA library was concentrated by ethanol precipitation (Materials and Methods 5.1). Following concentration the library was amplified using PCR, then subjected to the next round of separation as per the GELEX procedure set out above.

5.3.2 Cloning

After 3 rounds of GELEX the final DNA library was amplified for the last time using a Taq. Polymerase as described in Materials and Methods 5.3 and the recovered sequences are presented here:

Table 11 DNA sequences recovered from the GELEX process

Name	Aptamer Sequence
mba1	CCTGCTGATTCGTTGGGTGTTGTGTGGT
mba2	CCCGTANCGTACAGTCCCAGCTTGGTAT
mba3	CCCTGAGTCATAGTGTTCACGTGTT
mba4	CCACAACGAGCCGGGATTGCGGATTGCA
mba5	CCCTGAGTCATAGTGTTCACGTGTT
mba6	GGCGAATTGGGCCCCGAGGGGTCGACT
mba7	CCCGTANCGTACAGTCCCAGCTTGGTAT

mba8	GGCGAATTGGGCCCGAGTCGCATGCTCCC
mba9	CCGGTTGGGATGTGNCNCCCCNNATANN

After alignment the sequences produced from GELEX can be seen to show some consensus in positions 1,2,3,4, and 6, which indicates that the conserved region may be involved in aptamer binding. There is also a conserved area at bases 18 to 22 with again points towards the preservation of a motif, although there could be two populations of sequences with cytosine the more dominant over guanine in both of the “motifs”.

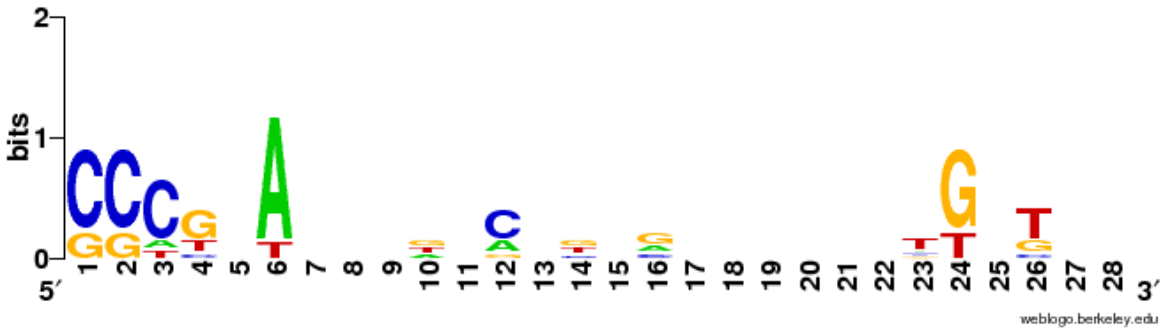


Figure 66 Sequence Logo of base occurance of recoverd MTase binding aptamer library. Sequence logos depict the relative frequency of each base at each position of the motif. The y axis indicates the information content measured in bits, and error bars represent standard deviations at each position due to the limited sample size.

Another interesting note is the comparison with the double stranded DNA Mtase binding region GAA(N)$_6$RTCG (Taylor *et al.* 1994, (Mernagh *et al.*, 1998). In two of the sequences, mba6 and mba8 , the binding sequences can be observed but with slight alterations in the number of bases between the 5’ and 3’ portions of the recognition sequences. In Mba 6 the recognition sequence may be amended to GAAN$_{14}$RTCG, and in mba8, GAAN$_{10}$RTCG, the striking resemblance to a DNA binding enzymes’ recognised binding site indicates that the DNA may be adopting a secondary structure that enables the single stranded DNA aptamer to mimic it.

Table 12 Comparison with mtase binding region GAAN6RTCG (Mernagh et al., 1998)

Mba1	CCTGCTGATTCGTTGGGTGTTGTGTGGT
mba2	CCCGTANCGTACAGTCCCAGCTTGGTAT
mba3	CCCTGAGTCATAGTGTTCACGTGTT
mba4	CCACAACGAGCCGGGATTGCGGATTGCA
mba5	CCCTGAGTCATAGTGTTCACGTGTT
mba6	GGCGAATTGGGCCCCGAGGGGTCGACT
mba7	CCCGTANCGTACAGTCCCAGCTTGGTAT
mba8	GGCGAATTGGGCCCGAGTCGCATGCTCCC
mba9	CCGGTTGGGATGTGNCNCCCCNNATANN

Recognition Sequence	GAANNNNNNRTCG

5.3.3 Dot blot of MTase Binding aptamer sequences

The recovered candidate sequences were assayed for binding to MTase using the dot blot procedure. Dot blot of MBA 1,2,3,4,5,6,7, 9, and a no DNA control from top to bottom against decreasing concentrations of MTase (10 µM, 5µM, 1 µM, 0.5 µM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, and 0) progressing from left to right, probed using streptavidin:HRP conjugate (0.125 ng/mL) and visualised using Fujifilm™ Las3500 Intelligent Dark Box.

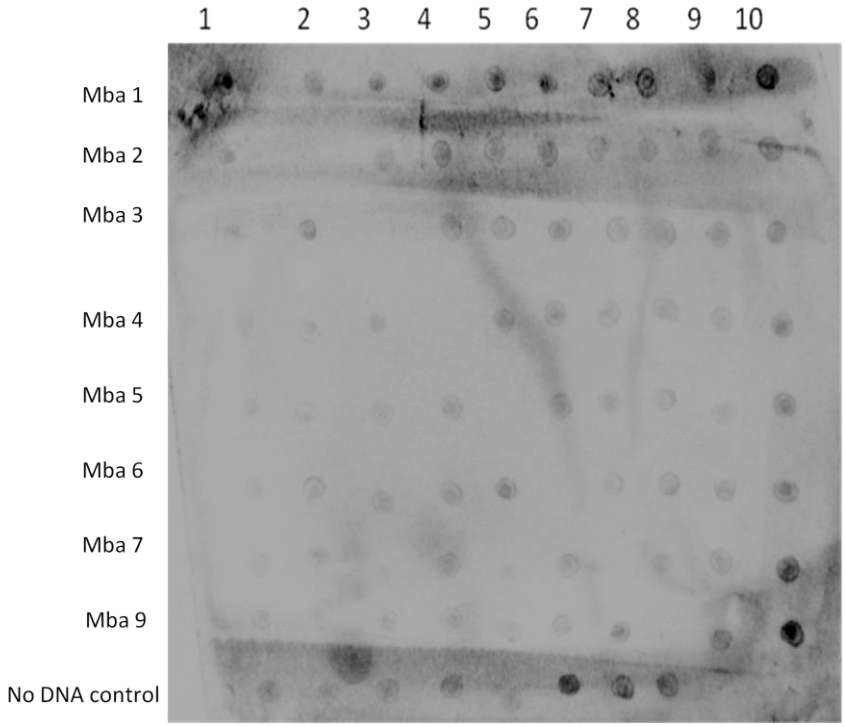


Figure 67 Dot blot of MTase Binding aptamer sequences. Dot blot of MBA 1,2,3,4,5,6,7, 9, and a no DNA control from top to bottom against decreasing concentrations of MTase (10 µM, 5µM, 1 µM, 0.5 µM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM, and 0) progressing from left to right, probed using streptavidin:HRP conjugate (0.125 ng/mL) and visualised using Fujifilm™ Las3500 Intelligent Dark Box.

The binding of the aptamers to MTase was not clarified by this method due to a large amount of non-specific binding, analysis using ImageJ (data not shown), a further method for MTase aptamer validation was sought.

5.3.4 Aptamer facilitated Protein sedimentation

The process of affinity purification is a well documented and widely practiced method of purifying proteins from solution by using a partner molecule with reversible binding to the desired target. Aptamer facilitated protein sedimentation is a variation of immuoprecipitation using aptamers immobilised on a surface. The aptamers are

biotinylated and bound to streptavidin that has been covalently bonded to Dynal® paramagnetic beads through proprietary chemistry. After significant washing, the beads are incubated with the target protein and unbound protein is removed. The bound portion can then be released using a high ionic strength buffer (such as 2 M NaCl) and examined using a denaturing SDS PAGE gel.

Using this technique it is possible to pull the target molecule out of solution by the magnetic attraction properties afforded by the beads. Although the beads are designed to stay in suspension they will sediment over time (>24hrs), this sedimentation can be exacerbated by centrifugation.

The method of pulling protein from solution can give some insight into the specificity of the protein for the target, if the aptamer does bind the target at all.

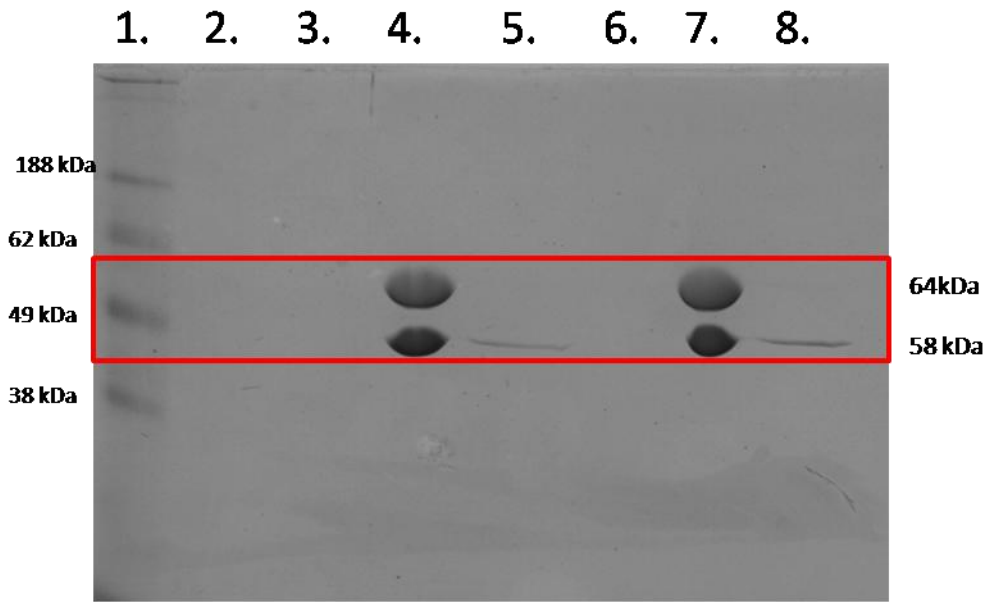


Figure 68 Aptamer facilitated pull down of Mtase from purified protein. Repeat of figure 4 10 % SDS PAGE gel of MBA 5 and MBA 9 facilitated Mtase pull down. Lane 1 See Blue Protein Ladder (Invitrogen), Lane 2 No DNA control Lane 4 MBA5 unbound fraction, Lane 5 Mba5 eluate, Lane 7 Mba9 Unbound flowthrough, Lane 8 MBA 9 Eluate . Such and such conc of aptamer was incubated with X conc of beads for X mins, washing etc.

Figure 68 is the SDS PAGE gel that resulted from the protein sedimentation with lane 5 and lane 6 representing the recovered protein whilst lanes 4 and 7 are examples of the unbound portion. The two bands observed in lanes 4 and 7 correspond to the co-purification partners of, HsdM and HsdS, EcoR124I subunits with the upper band corresponding to HsdM and the lower HsdS band. It can be then observed that although the aptamers do bind to a protein,

it is not the intended target, but the partner protein to which the aptamers have been raised. This can help to explain the resemblance of some of the sequences to the binding site desired by the specificity subunit.

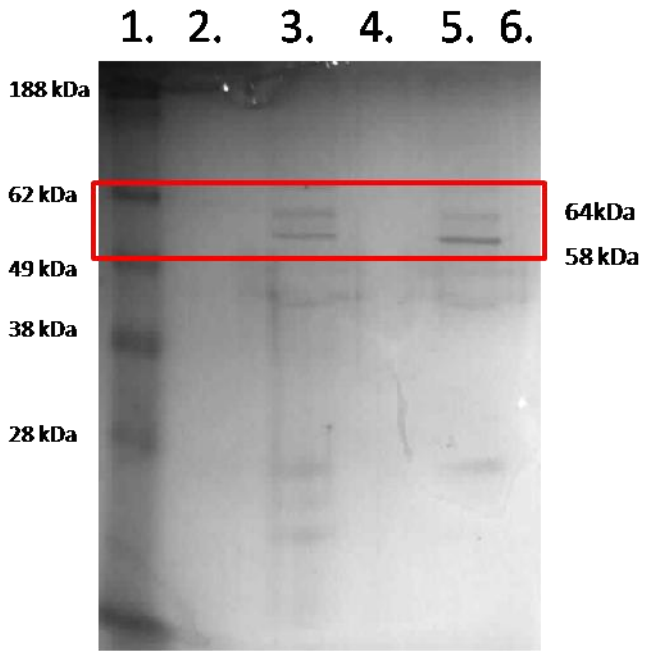


Figure 69 Aptamer facilitated pull down of MTase from crude lysate. 10 % SDS PAGE gel of MBA 5 and MBA 9 facilitated Mtase pull down. Lane 1 See Blue Protein Ladder (Invitrogen), Lane 2 MBA5 unbound fraction, Lane 3 Mba5 eluate, Lane 4 Mba9 Unbound flowthrough, Lane 5 MBA 9 Eluate, Lane 6 No DNA control

This method also lends itself the crude purification from a complex background. Figure 69 depicts the recovery of the desired protein from cell lysate. 500 mL of JM109 cells were grown overnight and gently lysed by incubation with a high salt buffer. The lysate was incubated with 5 µL of 10 mL mL^{-1} MyOne Dynal paramagnetic beads (Invitrogen UK) that had been prepared with biotinylated aptamer and followed the protocol described above. Although some other proteins have been purified it is postulated that they could be removed through a more stringent washing methodology.

5.4 HPLC SELEX

To complete the suite of laboratory based aptamer isolation methods a technique was sought that would be suitable for the enrichment of SELEX libraries against small molecule targets. Currently, SELEX methods directed to small targets require either the use of

capillary electrophoresis (CE-SELEX (Bowser, 2005)or NECEEM (Berezovski *et al.*, 2006) or the immobilisation of the target to an affinity matrix (Green *et al*., 1996; Jellinek *et a*l., 1994) or organic dyes (Ellington and Szostak, 1990,1992). High Performance Liquid Chromatography (HPLC) is a common laboratory technique based on the separation of molecules in a liquid phase, either by ionic charge, size, or other. The separation is achieved by the migration of species through a column of a solid matrix.

The proposed method relies on the fact that the small molecule target will migrate through the matrix at a higher rate than the random DNA library, and that a DNA:target complex will migrate at a further separate rate. Once complete separation can be assumed to have been achieved the fraction that is known to contain the complex can be isolated and amplified as in regular SELEX. Again the enriched library will be incubated with the target, and separated a second time, and the process repeated until the library is sufficiently enriched before cloning and identification of candidate aptamers.

Any fraction found to absorb light at 450 nm and elute away from the known elution time associated with the unbound NT fraction can be considered to contain NT. This fraction can then be amplified as per the SELEX procedure.

5.4.1 Results

Figure depicts the four chromatograms drawn from NT SELEX, **A** shows the migration of NT with a peak found at approximately 1.25 minutes from the void volume. With only NT present and the detector set to λ450 nm it can be confidently assume that 1.25 minutes is where NT will elute. **B** is a chromatogram of the elution profile of the 1 µM random naked DNA library at λ260 nm, this shows a broad profile with a large peak at 6 minutes which can be assumed to be the DNA library. The other earlier peaks present are most likely incomplete synthesis products, and although they may interfere with binding by providing false motifs, they will not be selected for in subsequent rounds of SELEX due to incomplete sequences which would most likely mean the absence of priming regions. **C** is a demonstration of the absorbance of NT at λ260 whilst **D** is the λ450 nm chromatogram depicting the elution profile of NT and the random DNA library after incubation together. **E** is λ260 nm representation of **D** with a large peak that occurs at 1.25 minutes and another that occurs at 6 minutes, which could be considered to be the elution of NT and the library

respectively. The overlay **F,** however, highlights a shift of the λ450 nm NT profile upon the mixing with the DNA library. This would appear to be the formation of a complex.

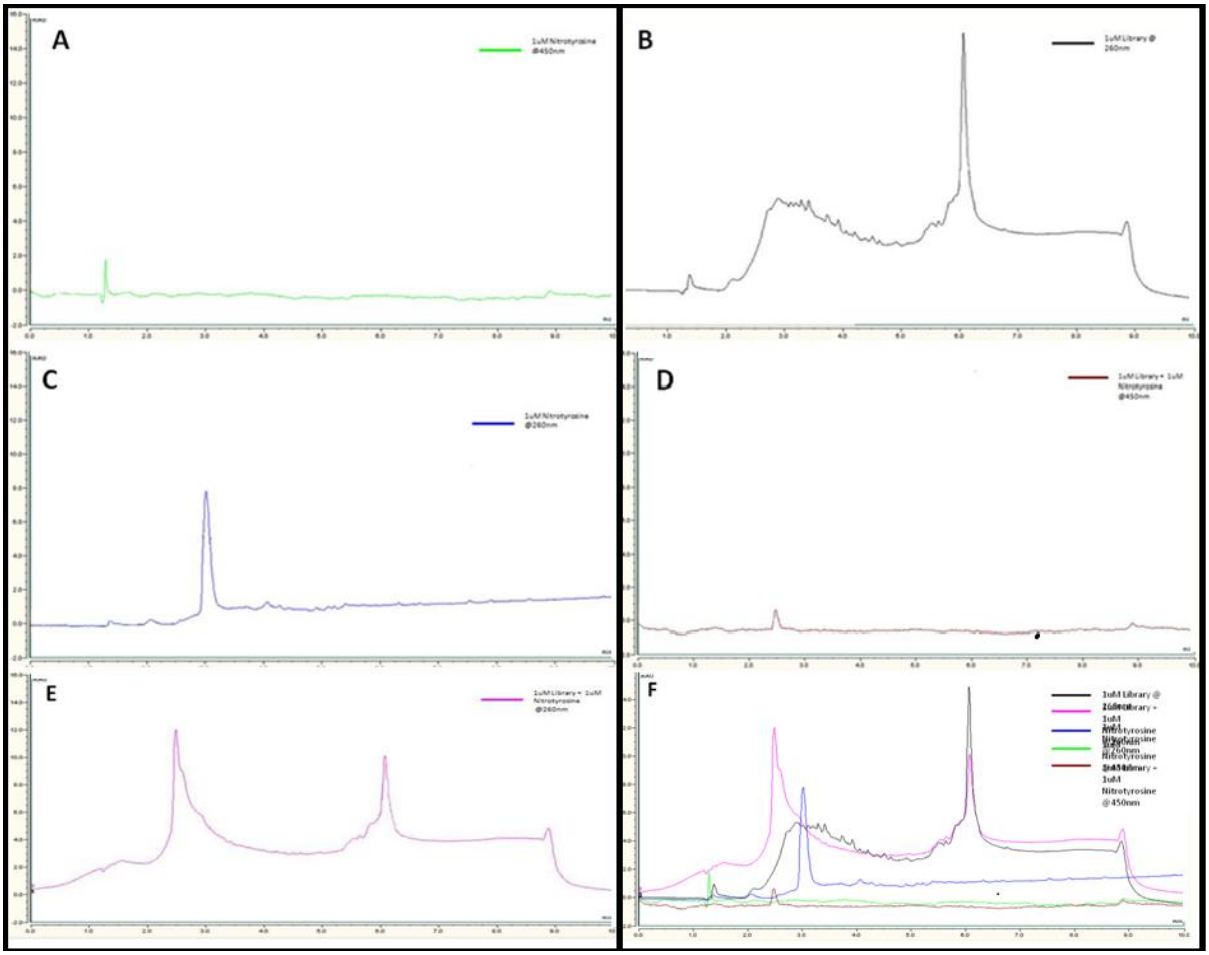


Figure 70 Chromatograms depicting HPLC SELEX of Nitrotyrosine. Chromatograms produced using Dionex LC2000 HPLC machine, with a DNApac PA200 column, samples run for a total of 20 minutes at a flow rate of 1 mL min-1. Section A is a chromatogram of Nitrotyrosine with detection at λ450 nm, Section B is a chromatogram of the naked DNA library with detection at λ260 nm, Section C is a chromatogram of Nitrotyrosine with detection at λ260 nm, Section D is a chromatogram of the combination of nitrotyrosine incubated with the random library with detection at λ450 nm, E is a chromatogram of the combination of nitrotyrosine incubated with the random library with detection at λ450 nm, F is an overlay of chromatograms A, B, C, D, and, E.

5.4.2 Cloning

The isolated sequences were cloned in to pGEM-T-Easy as defined in Materials and Methods 5.3.

5.5 Q-Nano DNA electrophoresis

The concepts introduced in this chapter investigate the isolation of aptamer libraries using processes that partition the random library from the bound target:DNA complex through alternative separation methods. The qNano (Figure 71) nanopore provides a suitable platform for the dynamic separation of molecules by size (Sowerby *et al* 2007). The tuneable

aperture can be adjusted, giving a nanopore that range from 10-100 nm in size. By immobilising the target to a surface of a particle that is too large to translocate through the aperture, the non-binding species can be removed.

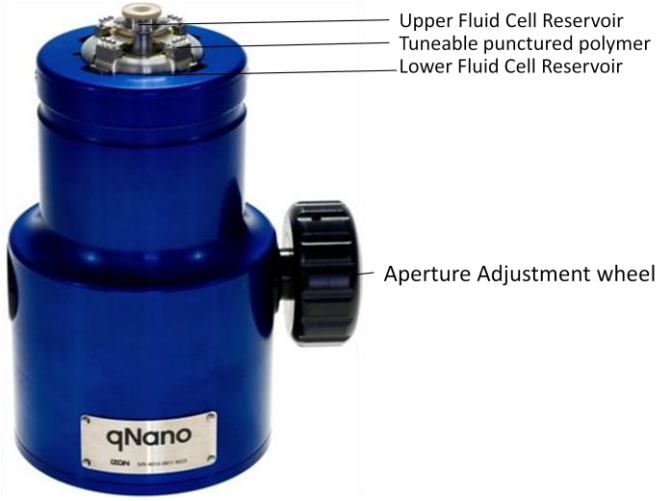


Figure 71 qNano Scanning Ion Occlusion Sensing Platform. Image of the qNano nanopore sensing platform

Through the attachment of biotinylated Human Rhinovirus 14 3C to streptavidin conjugated MyOne paramagnetic beads the DNA library is incubated with the target, then separated using the electrophoretic force applied across the nanopore. After sufficient time under the field the aperture can be closed, and the partitioned components recovered. The bound species are then eluted from the target and amplified and the process repeated until the library can be considered to be sufficiently enriched prior to identification of the sequences by cloning and sequencing.

5.5.1 Analysis of translocation by qPCR

The dynal beads have streptavidin immobilised on the surface through proprietary chemistry. HRV143C has been produced that carries a biotin moiety (article submitted) which will bind to the streptavidin with a high affinity (Weber *et al.* 1989). Using this interaction as an anchor point allows the confident separation of non-biotinylated species through methods such as electrophoresis. In this experiment the random sequence DNA starting library was introduced to the upper reservoir of the qNano instrument. After a series of intervals, small aliquots were taken from the lower reservoir was undertaken. Each aliquot was then subjected to PCR to amplify any of the target sequence present. The

amount of amplified product, will allow the estimation of speed of translocation, and then allow further extrapolation to the amount of time that is needed for the SELEX process.

5.6 qNano SELEX

100 µL of the SELEX random library (Materials and Methods 7) was incubated with 30 µL of 10 mg/mL MyOne streptavidin coated paramagnetic Dynal beads that had been pre-incubated with biotinylated HRV14 3C and incubated in electrolyte buffer (0.1 M KCl, 10 mM Tris pH 8, 0.01% Triton X-100 (Willmott *et al.*)). After 10 minutes at room temperature the bead:DNA mixture was introduced to the top reservoir of the qNano system. With the aperture of the nanopore set to 100 nm and the beads reported to have an average of over 1 µm in diameter (Invitrogen) the majority of the beads can be said remain in the upper reservoir. After 4 hours under 170 mV potential the upper and lower reservoir were collected. The lower reservoir was concentrated using QIAquick PCR cleanup columns and prepared for cloning.

5.7 Cloning

Sequences were cloned, expressed and isolated according to protocol (Materials and Methods 5.3) and the following sequences were recovered (Table 13).

Table 13 DNA sequences recovered from qNano SELEX

Sequence Name	DNA sequence
qNanoHRV143C1	**TGTCTATGCAATAGTCGTTGGAGATAGTT**
qNanoHRV143C2	**GCCATGATGAGCATGCTTGTCCTAGACCG**
qNanoHRV143C3	**GCACCCACTAGGCGGGGAGCAGTACTCC**

Unfortunately this method only yielded three novel DNA sequences, which, when compared to each other appear to be completely random. This allows us to surmise that although the procedure may have been sufficient to produce novel aptamers the method on this occasion was flawed. Pressure on time prevented the complete examination of the technique, but it can be seen as pilot study in much need of further investigation.

Table 14 ClustalW output of multiple alignment of qNanoPPBA sequences against HRV143CBA sequences

1	----------CCCACACACTACTCTGCCGATTTGGT-- 26	22	-------CCCAAT-GAACATGCCACAGTTGGTCGT---- 27
14	----------CCCACACACTACTCTGCCGATTTGGT-- 26	48	-------CCCAAT-GAACATGCCACAGTTGGTCGT---- 27
17	----------CCCACACACTACTCTGCCGATTTGGT-- 26	7	-------CCCAAT-GAACATGCCACANTTGGTCGT---- 27
25	----------CCCACACACTACTCTGCCGATTTGGT-- 26	42	-------CCCAAT-GAACATGCCANANTTGGTCNT---- 27
38	----------CCCACACACTACTCTGCCGATTTGGT-- 26	31	-------CCCAAT-GAANATGCCANANTTGGTCNT---- 27
50	----------CCCACACACTACTCTGCCGATTTGGT-- 26	32	-------CCNANCAAAAGGTGCNATGCTTTACN------ 26
56	----------CCCACACACTACTCTGCCGATTTGGT-- 26	43	-------CCNANCANAANGTGCNATGCTTTACNGN---- 28
57	----------CCCACACACTACTCTGCCGATTTGGT-- 26	23	-------CCGAGCAGAAGGTGCGATGCTTTACGGT---- 28
58	----------CCCACACACTACTCTGCCGATTTGGT-- 26	19	-------CCCATCGACA-GT-CTGCTTCAGATCGGT--- 27
68	----------CCCACACACTACTCTGCCGATTTGGT-- 26	39	-------CCCATCGACA-GT-CTGCTTCAGATCGGT--- 27
70	----------CCCACACACTACTCTGCCGATTTGGT-- 26	qNanoHRV143C2	----GCCATGATGAGCATGC-TTGTCCTAGACCG----- 29
75	----------CCCACACACTACTCTGCCGATTTGGT-- 26	53	-------CCAAGCGGGCGCGAGAACAACGTACGGT---- 28
16	-------CCAAGCGGGATAC-ACACTACTGGGACGC--- 28	66	-------CCAANCGGGCGCNAGAACAACGTACGGT---- 28
24	-------CCAAGCGGGATAC-ACACTACTGGGACGC--- 28	5	---------AGGCGGGGGGACTGACAATGTG-------- 22
63	-------CCAAGCGNNATAC-ACACTACTGANANNC--- 28	11	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
18	----CCTCGGGACGGTGCTTCACCCTGCTGGT------- 28	12	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
26	----CCTCGGGACGGTGCTTCACCCTGCTGGT------- 28	27	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
2	--CACCTCGGGACGGTGCTTCACCCTGCTGGT------- 30	49	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
30	-CCCACAGACATTANGACGCTACATGGGGT--------- 29	51	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
41	-CCCACANACATTANGACGCTACATGGGGT--------- 29	74	--CCCGGGCGGACAGCAGGCTGTTCGTTGC--------- 28
76	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	15	--CCCGGGCGGACAGCAGGCTGTTCGTTG---------- 27
73	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	33	-------CCAACCGGAATCCAGTCGAGGT---------- 22
72	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	47	----CCGGCGGGCGGGAGGCATCTGAGGGAGT------- 28
69	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	qNanoHRV143C3	GCACCCACTAGGCGGGGAGCAGTACTCC---------- 28
59	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	4	----------CCACATTGTCCTTCCTTGTGTGT----- 23
55	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	8	----------CCACATTGTCCTTCCTTGTGTGT----- 23
52	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	54	----------CCCATGACCTGCGTTCATCG-TGTTCGGT 28
46	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	61	----------CCGGCTGTGCTTTTACTGGTGTTGGGT 27
45	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	44	-------TCGTGCGTAACGTGTGCTTGGTGGT------- 25
40	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	65	----CCATCGT-CGTNAATTGTCTTCNGTGGT------- 27
36	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	29	-----CGTTTT-CTTGATGTTTTTGCATTGGT------- 26
28	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	9	----------CATGGCAGGG---TGCANTTGTGGT---- 22
21	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	35	-------CTCTATGGCAAGCGT-TGTGTTTGTGGT---- 27
20	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	60	-------CCCGGGAATAACCGTACGAGTTCGGGGT---- 28
10	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	qNanoHRV143C1	----TGTCTATGCAATAGTCGTTGGAGATAGTT------ 29
3	-CCCACAGACATTAGGACGCTACATGGGGT--------- 29	62	----CCGGGGCACACT-GACGGGACTAACAGCGCGC--- 31
71	-CCCACAGACATTAGGACGCTGCATGGGGT--------- 29	67	----CCGGGGCACACT-GACGGGACTAACAGC------- 27
64	-CCCACATACATTAGGACNCTACATGGGGTGCGC----- 33	34	----CCTGTGCACACTTAATGTTGCGTTCGGT------- 28
37	------CCTCATCAGTAGTCATCATCGT---------- 22		
6	-------CCCAAT-GAACATGCCACAGTTGGTCGT---- 27	Consensus	--CCCACAGCCATCAGGACGCTACACGGTGGGTTTGGTGT
13	-------CCCAAT-GAACATGCCACAGTTGGTCGT---- 27		

5.8 Conclusions

Overall the each of the three aptamer isolation methods presented in this chapter produced novel sequences, although the apparent low yields of aptamer sequences from the enriched highlight a need for further investigation.

The aptamer facilitated pull down of MTase from solution to purified protein (Figure 68) appears to be a suitable method of assaying overall aptamer binding and the specificity of putative aptamer sequences. Although the aptamers preferentially bound the target protein's purification co-partner, the method would highlight the specificity of the aptamer and indicate a user error rather than an issue with the method. When the aptamers are probed against a complex mixture in the form of the crude cell lysate the two aptamers appear to perform badly. Upon further examination however it can be deduced that the purification is ~90% of the target MTase and HsdS subunit with the contaminants a carryover from the purification process. Further work indicates that the contaminants are avoidable with further, more stringent washes, (data not shown) but this has an impact on the yield of protein. In conclusion, the method is suitable for the screening and analysis of aptamer binding and specificity, but the result is hard to quantify due to the inconsistency of using paramagnetic beads. As some of the bead volume is lost through the coarse washing involved in removing non specific binding proteins, a discrepancy arises from experiment to experiment. This could possibly be overcome with the immobilisation of the aptamers to a column with a known and consistent volume.

The novel process of library enrichment using Dionex 3000LC HPLC machine can be considered a success because N novel sequences have been isolated, but the proposed aptamers lack a suitable assay to determine aptamer binding. Possibly, this could be overcome by conjugation of the sequences to the interior of an aperture of a nanopore(Gu L. Q., Shim J.W. 2010), for if the target or the aptamer sequences were bound the translocation profile and dwell time of the analyte may be slowed and this signal analysed to give an indication of any binding activity. An attempted experiment to define the nature of the aptamer to nitrotyrosine binding for the candidate aptamers using an affinity matrix with the target molecule perfused throughout an agarose gel has been attempted (not shown here), but the experiment failed due the overall negative charge of NT which

migrated in the along with the DNA. This made it difficult to determine any retardation of the aptamers that could be due to binding with NT.

All three methods of aptamer separation presented here are examples of methods of aptamer library enrichment that can be attempted by most adequately equipped molecular biology laboratories. The techniques demonstrated here do not require specific and expensive equipment and can produce aptamers for a variety of targets. Nanopore selection may further be advanced by a variation upon the method presented here, where the target is immobilised to the rim of the nanopore which would provide the potential to give real time analysis of the proposed aptamer binding kinetics.

These methods all represent the potential of aptamers as a complement and alternative to antibodies in a variety of applications.

6 Aptamers within biosensors: A Novel Sandwich ELISA based Molecular Amplifier

Along with many other parallels with antibodies, the use of aptamers in biosensors as a molecular recognition element is highly advocated (Tombelli *et al.*, 2006). Within this chapter the aim is to demonstrate modifications on known biosensing systems, and to highlight the applications and benefits of aptamers through substitution with antibodies.

6.1 The Molecular Amplifier

A potential problem that can occur in the development of a sensitive biosensor is the ability to accurately detect signals that can be considered to be below the conventionally received detection limits (Cho *et al.*, 2009). In order to truthfully amplify the signal whilst keeping false positives and noise to a minimum, a method with high specificity is required. The molecular amplifier facilitates this by forming a modified ELISA sandwich (Figure 72) around the antigen or target molecule and by utilising the ability of streptavidin to bind up to four biotin molecules amplify the signal (DeChancie & Houk, 2007).

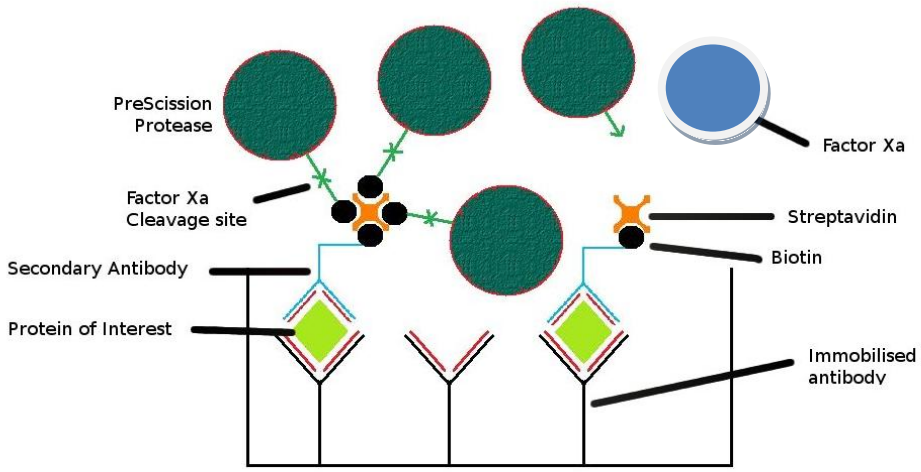


Figure 72 Construction of Modified Sandwich ELISA. A schematic diagram of the construction of a modified Sandwich ELISA.

The sandwich, is formed by a primary antibody that is immobilised on an activated surface. Subsequently, the antigen is introduced to the immobilised antibody which, if specific, will then form a complex together. Following the removal of any unbound protein a secondary antibody specifically binds to an unoccupied epitope of the target protein. As in some

traditional ELISA technology the secondary antibody is conjugated with a streptavidin molecule to produce an attachment point for 1-4 biotinylated molecules. The streptavidin provide a flexible attachment point to which any molecules with a biotin moiety can be easily and reliably attached. In the case of the molecular amplifier system the streptavidin is occupied by the protease Human Rhinovirus 14 3C (HRV143C) which then cleave a peptide bond that releases a further protein (Figure 73). This further protein can be any protein with the correct peptide recognition sequence, for example the motor protein HsdR to further downstream applications or eGFP for quantification of the release efficiency.

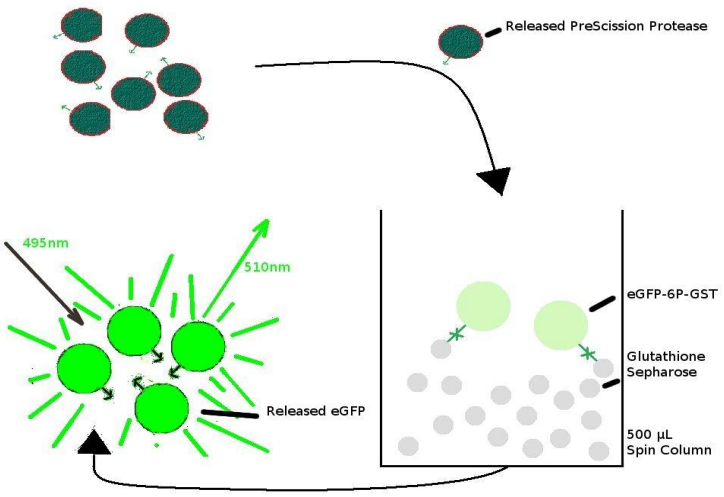


Figure 73 Human Rhinovirus 14 3C mediated eGFP release. Schematic diagram of eGFP release upon specific cleavage of a peptide bond

6.2 HRV143C mediated release of eGFP

6.2.1 eGFP spin column preparation

Microcentrifuge spin columns were constructed from G-25 spin columns (GE Healthcare®, Amersham, U.K) by application of 300 µL of Glutathione Sepharose™ 4 Fast Flow (GE Healthcare®, Amersham, U.K) then spun in a micro centrifuge at 1000 x g for 1 minute. The column was washed by re-suspension in 300 µL dH2O, followed by a spin in a micro centrifuge at 1000 x g for 1 minute and repeated three times. The column was then equilibrated by re-suspension in 300 µL of HRV143C cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5) then spun at 1000 x g for 1 minute and repeated three times. 100 µL of 10 µM eGFP-GST (~54 kDa) was applied to the column and allowed to bind to the media incubate at room temperature for 10 minutes. Any

unbound protein was removed by a 1 minute spin at 1000 x g then equilibrated with three 300 µL HRV143C cleavage buffer washes.

6.2.2 eGFP Release and quantification

The eGFP-GST fusion protein contains a HRV143C cleavage site for release of the eGFP portion. The HRV143C released from the ELISA sandwich by Factor Xa was applied to a pre-prepared eGFP glutathione sepharose column then incubated at 4 ^{o}C for 4 hours. After incubation eGFP released from by HRV143C digestion was quantified by fluorescence using a BMG LABTECH OPTIMA POLARstar™ plate reader using 490 nm excitation wavelength and 510 nm emission detection.

6.2.3 eGFP Calibration Curve Construction

In order to determine the potential limit of detection for eGFP a calibration curve was constructed measuring the amount of fluorescence detected at 510 nm at pre-determined concentrations (Figure 74). Extrapolations from Figure 74 indicate that detection becomes limited at concentrations below 1 µM when compared to the higher (79 µM to 5 µM) concentrations; a second curve was assembled at the lower concentrations (Figure 75).

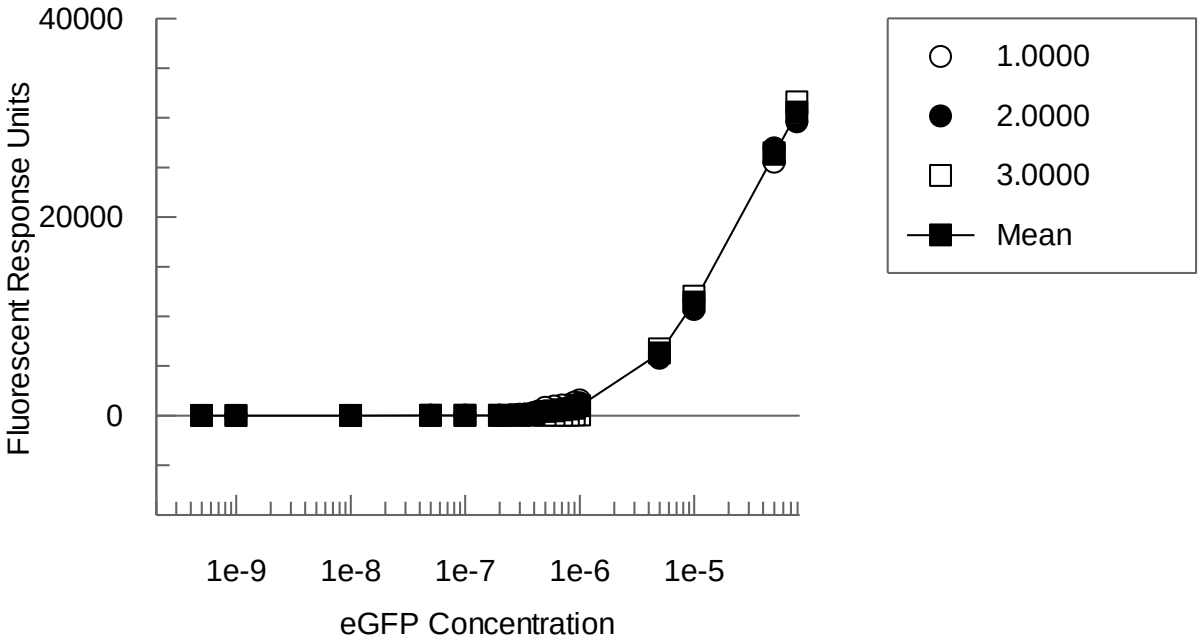


Figure 74 Calibration Curve of eGFP concentrations to determine limit of detection. Concentration dependent eGFP response calibration curve Decreasing concentrations of eGFP dissolved in HRV143C cleavage buffer were analysed using a BMG LABTECH OPTIMA POLARstar™ Filters used: emission 490 nm, excitation 510 nm and arbitrary fluorescence response units used.

Detection of eGFP by fluorescence can give concentration estimates down to approximately 5 µM, from Figure 74 however a signal is detectable to 1 µM. For a more detailed grasp on the lower concentrations a second curve was constructed that allows displays the lower fluorescent response in greater detail. From this graph (Figure 75), it is possible to deduce with some confidence that the lower level of detection is approximately 500 nM, any response below 500 nM eGFP cannot be confidently discerned from background noise.

The overall fluorescence emission spectra can be drawn to produce a roughly sigmoidal curve of observed response units (Figure 77). This curve allows the accurate prediction of eGFP present in a solution following extrapolation from a curve.

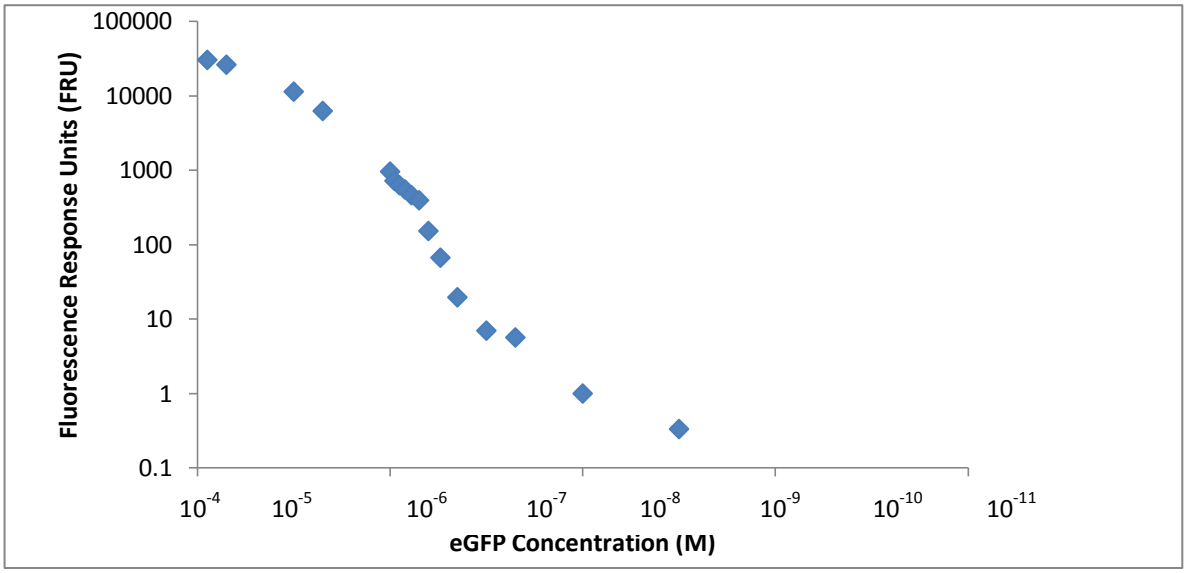


Figure 76 A titration of fluorescence emission of eGFP protein. Fluorescent output from a dilution series of the purified eGFP protein was determined as described (Materials and Methods) and plotted against a logarithmic plot of protein concentration. However, as can be seen the relationship between fluorescence and protein concentration is not linear and even appears somewhat sigmoidal.

6.2.4 HRV143C mediated release

After establishment of eGFP concentration dependant fluorescence and the standard curve that can be drawn from the result, a second set of data is needed to determine the efficiency of GST-eGFP cleavage by HRV143C. Earlier experimentation showed that an excess of HRV143C will cleave it's substrate within 4 hours, therefore an on-column cleavage using HRV143C and bound eGFP-GST as the substrate over 4 hours was undertaken. By simulating the release conditions of the second part of the modified ELISA sandwich it may be possible to predict eGFP release from the GST fusion and extrapolate the concentration of HRV143C from this. From the results (Figure 77) it can be seen that the limit for detection of concentration is ~1 µM HRV143C in 100 µL of HRV143C cleavage buffer applied to the column. The fluorescent response units have been converted to a percentage of the top concentration (in this case 10 µM HRV143C to minimise error and allow comparison between repeat experiments and limit the effect of degradation of the eGFP.

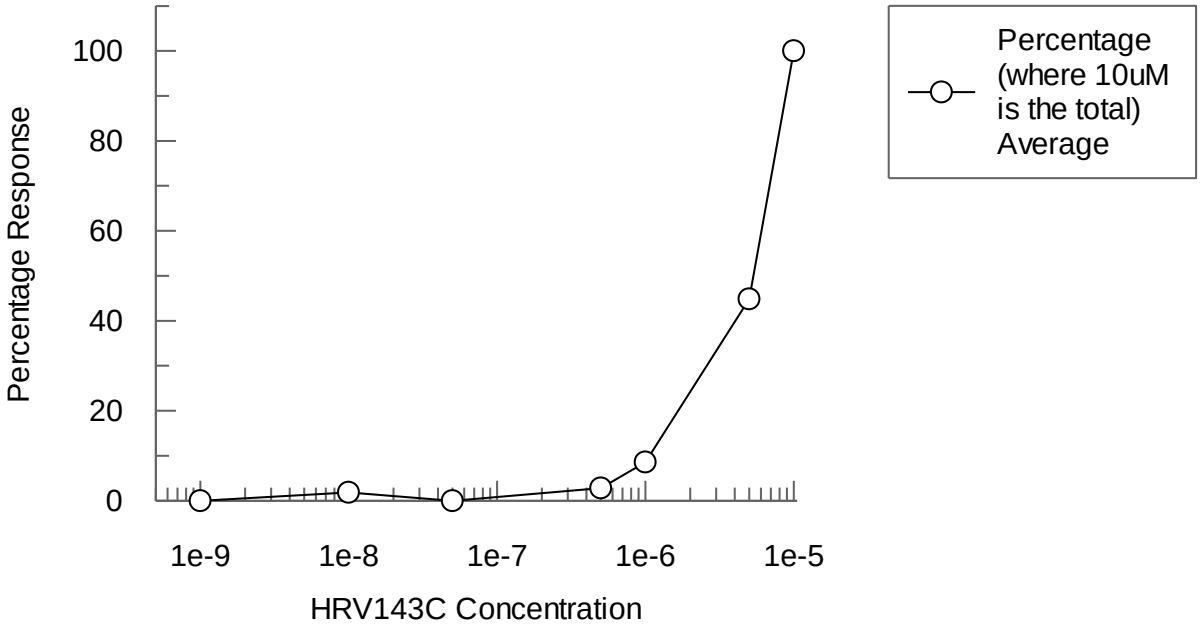


Figure 77 HRV143C controlled eGFP release Calibration Curve expressed as a percentage. eGFP released from column by HRV143C cleavage expressed as a percentage of the highest concentration. Detection of eGFP released from column by HRV143C in HRV143C cleavage buffer analysed using a BMG LABTECH OPTIMA POLARstar™. Filters used: emission 490 nm, excitation 510 nm and arbitrary fluorescence response units used.

As the concentrations of HRV143C are based on a logarithmic scale a simple transformation of the x axis provides a scatter graph that gives a linear progression of fluorescence response (Figure 78.). This indicates that the fluorescent response is concentration dependent and directly related to the concentration of HRV143C applied to the column. Again, the results indicate that the sensing of low concentrations of HRV143C is difficult below 5 µM with a detection limit of ~1-2 µM for a reliable extrapolation of HRV143C concentration.

Further investigation into the efficiency of 1-10 µM HRV143C cleavage (Figure 78) corroborate the earlier concentration mediated release of eGFP from the column (Figure 77) and fit around the trend line drawn from the mean percentage decrease, this provides a useful tool for estimating the amount of eGFP, and subsequently, HRV143C concentration within the 100 µL eluent of a column.

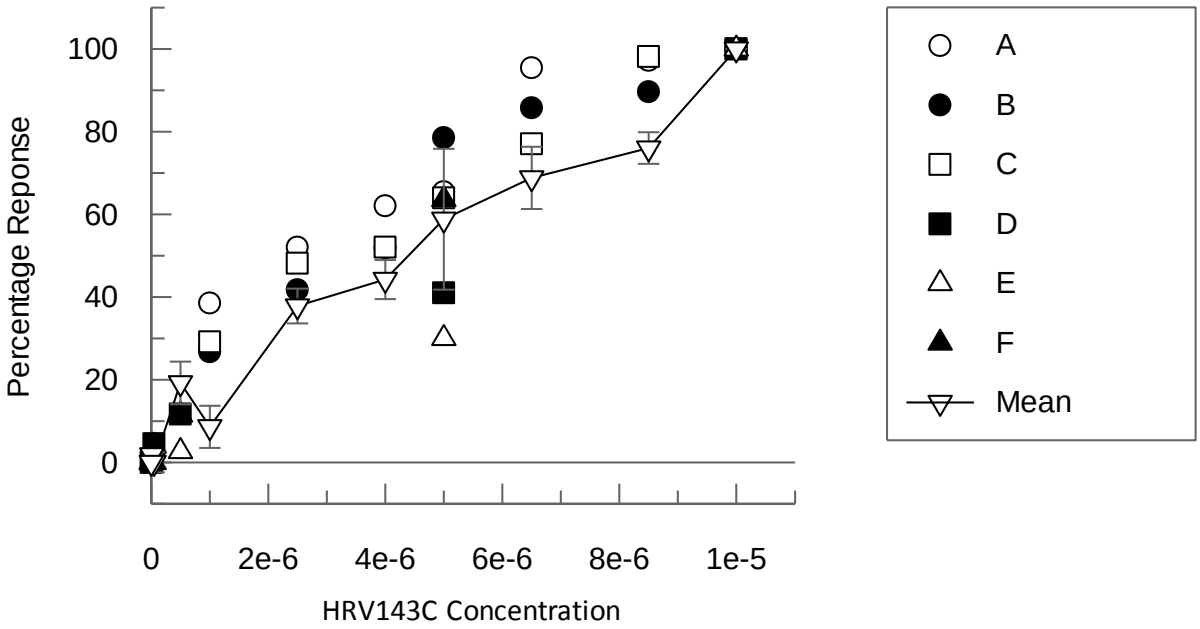


Figure 78 eGFP Release Calibration Curve. A graph summarising repeats of eGFP released from column by HRV143C cleavage expressed as a percentage of the highest concentration with extra points for mid µM range. Detection of eGFP released from column by HRV143C in HRV143C cleavage buffer analysed using a BMG LABTECH OPTIMA POLARstar™. Filters used: emission 490 nm, excitation 510 nm and arbitrary fluorescence response units used.

6.2.5 Sandwich ELISA construction

The ELISA sandwich is assembled by the addition of known decreasing concentrations of a nitrotyrosine containing protein standard to the surface pre-immobilised primary antibody, followed by the biotinylated secondary antibody (Hycult Biotech®, Uden, The Netherlands). each step interspersed with one hour long incubations at room temperature. To each well 100 µL of 2 µM streptavidin (Thermo Fisher Scientific®, Loughborough, U.K.) and incubated for 1 hour at ~23 ^{o}C then washed with 1x Phosphate Buffered Saline (PBS). After washing, 100 µL of 12 µM biotinylated Human Rhinovirus 14 3C [GE Healthcare®, Amersham, U.K]) and incubated for 1 hour at ~23 ^{o}C. Following washing, the complete sandwich was incubated with Factor Xa (GE Healthcare®, Amersham, U.K) in Factor Xa cleavage buffer (20 mM Tris-HCl pH 8.0,100 mM NaCl, 2 mM $CaCl_2$) for 16 hours at ~23 ^{o}C.

6.2.6 Molecular Amplifier ELISA sandwich

Following the creation of calibration curves and the completion of the eGFP on column release data the full ELISA sandwich was constructed (Figure 72 and Figure 73). The full experiment relies on each sub-section of the sandwich binding and releasing properly and

uses the assumption that in 4 hours one molecule of HRV143C will cleave and release one eGFP molecule from the eGFP-GST fusion. The amplifying effect of the streptavidin is caused by the availability of the three vacant biotin binding sites of the surface bound streptavidin being taken up by an excess of biotinylated HRV143C. Assuming complete release of the HRV143C from the surface bound ELISA sandwich, there should be an increase in signal from the original protein standard concentrations of approximately 2-3 fold. Using this method it should be possible to detect concentrations lower than the limit of detection of the eGFP release curve (Figure 77) from ~1 µM to theoretically 500 nM to 250 nM.

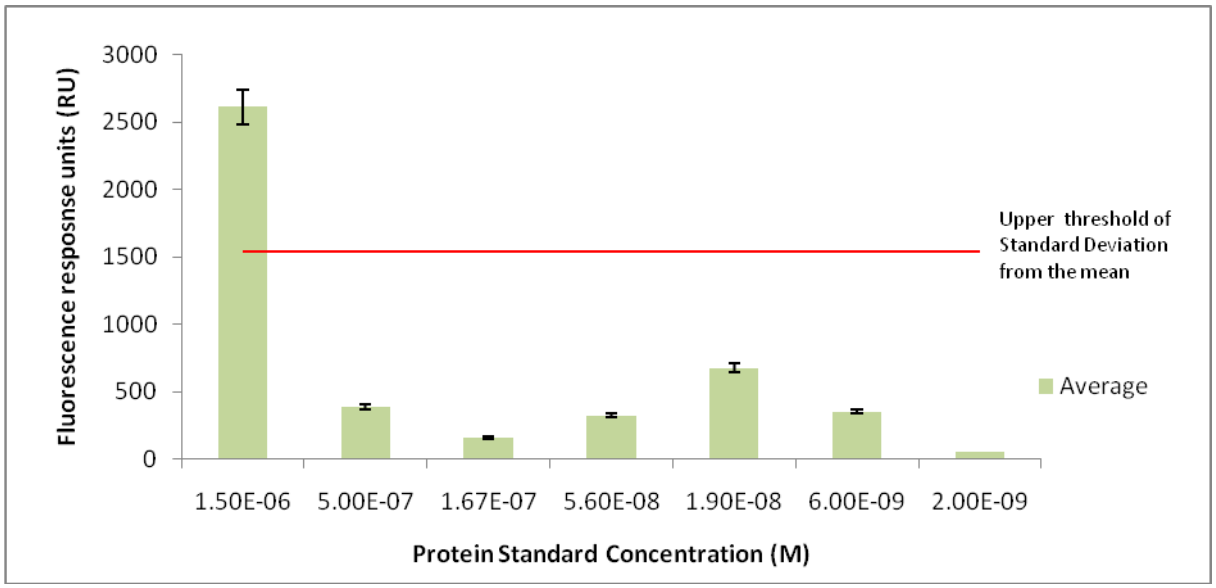


Figure 79 Molecular Amplifier final results. Molecular Amplifier final results. Accumulated, blank adjusted, mean fluorescent response results of eGFP release from GST fusion via 4h HRV143C cleavage, following 16h release of HRV143C from ELISA sandwich by Factor Xa. Error bars show standard error.

From the final results graph (Figure 79) it is possible to draw the conclusion that only the 1.5 µM protein standard concentration can confidently be detected by this method. Although the results are blank adjusted the red line indicated the upper threshold of the standard deviation and therefore the only reliable and confident conclusion is that anything that lays below that line cannot be accepted. Although there may be an amplification event it is not possible to divorce this from natural eGFP-GST decay causing the either the eGFP fusion to auto-cleave or dissociation from the Glutathione sepharose column.

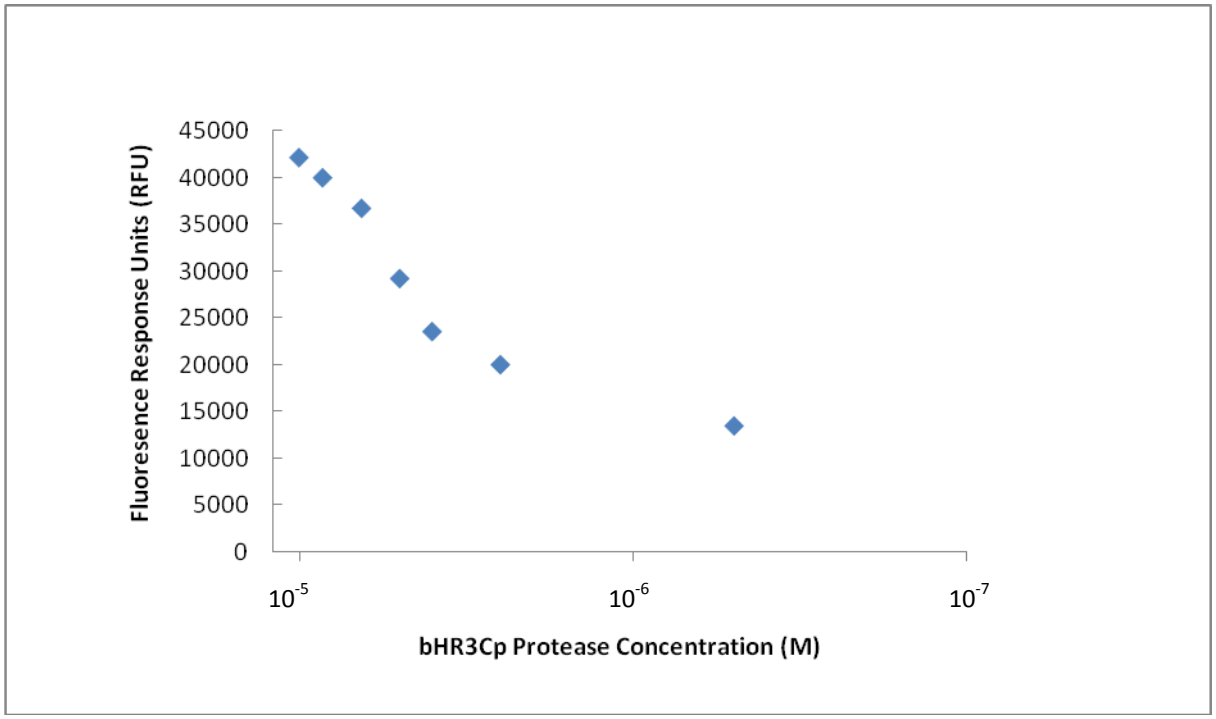


Figure 80 **Fluorescent detection of eGFP release from a column by bHR3Cp protein. The GST-(pp)-eGFP fusion protein was immobilized on a glutathione column and activity of the bHR3Cp protease was determined by release of eGFP, measured as fluorescent output, through cleavage at the bHR3Cp (pp) cleavage site. The bHR3Cp protease demonstrates activity on this column based assay down to micromolar concentrations.**

6.3 Enzyme Linked OligoNucleotide Assay (ELONA)

The use of antibodies as molecular recognition elements is well documented (Bang *et al.*, 2005, Centi *et al.*, 2008, Deisingh, 2006, Mairal *et al.*, 2008), it stands to reason therefore that if aptamers are to be considered true replacements or alternatives to antibodies the substitution of one for the other should be achievable. The Enzyme Linked ImmunoSorbant Assay provides a useful, quick and cheap way of assaying proteins in solution. Through the use of primary and secondary antibodies, a protein, or other molecule, can be captured and probed. The three common methods of probing for the secondary antibody are fluorescence, chemiluminesence, and colourmetric. For each of the methods the signal is produced by a chemical group that has been conjugated to the secondary antibody. It has already been stated that, due to the methods of synthesis of nucleic acids, the attachment of these (and other) groups is readily attainable in high quantity and cost-effectively.

The Enzyme Linked OligoNucleotide Assay (ELONA) is a method where the ELISA model has been altered to incorporate the use of primary and secondary aptamers rather than the traditional antibodies (Figure 81).

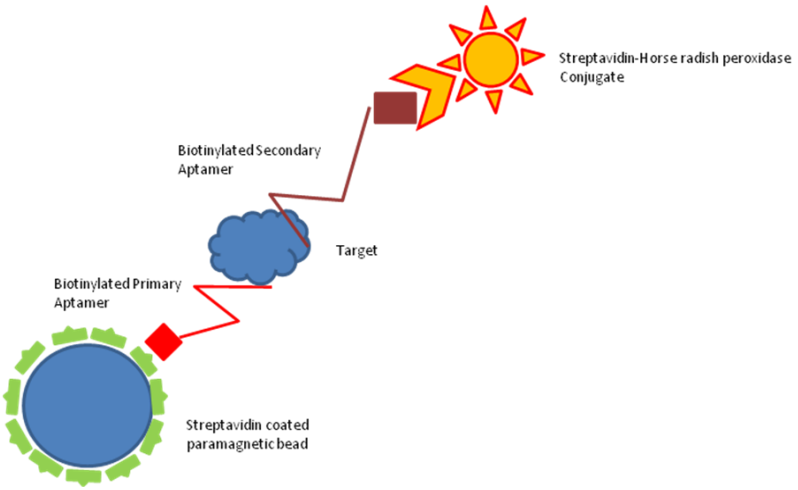


Figure 81 Schematic diagram of ELONA. Diagram of the adjucsted ELONA sandwich. Biotinylated Aptamer 1 is bound to a streptavidin coated paramagnetic bead, aptamer 1 binds the target molecule. Aptamer 2 selectively binds to the same target but at an alternative epitope. Aptamer 2 is conjugated with a streptavidin Horseradish Peroxidase molecule which can be detected using chemiluminescence.

The ELONA were constructed as follows: 5 µL 5 ug/mL streptavidin coated paramagnetic beads (MyOne beads, Invitrogen) were washed 3 times with 1 mL 1 x PBS. The beads were then incubated with 1 mL 1 µM of the designated biotinylated primary aptamer for 2 hours at 4^{o}C. The unoccupied biotin binding sites were then blocked by a blocking solution of

150 µL 1 ug/µL Biotin in 1 x PBS. The beads were then washed with 1 mL1 x PBS a further 3 times. The aptamer activated beads were then incubated with 1 µM MTase for 2 hours at 4^{o}C and then washed with 1 x PBS as before. The beads were then incubated with 1 mL1 µM of the assigned secondary aptamer for 2 hours before a washing step with 1 mL 1 x TBST 3 times. Finally, the beads were incubated with 1:5000 dilution HRP streptavidin conjugate (Invitrogen) in 1 X TBST 2 hours. The beads were then washed with 1 x TBS 3 times. Before development of the ELONA as described for the dot blot method in (Materials and Methods 5.1) imaging on Fujifilm Intelligent Dark Box reader.

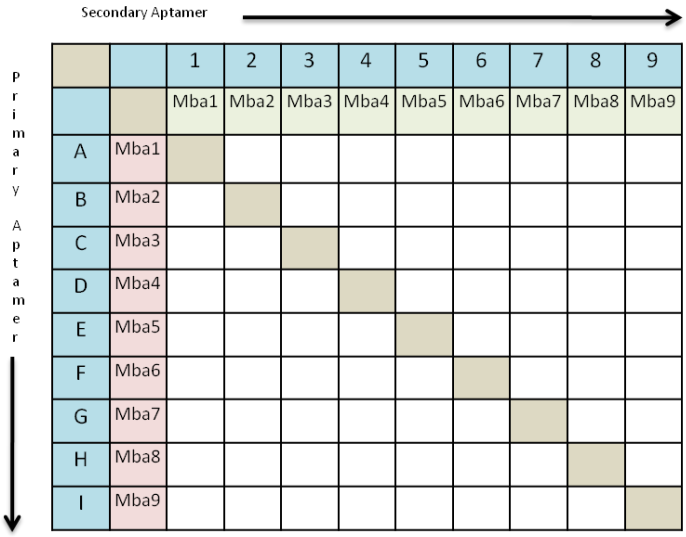


Figure 82 Diagram of the aptamer layout for MBA ELONA. Table to display the locations of each primary (A-I) and each secondary (1-9) aptamer to construct a suitable ELONA sandwich.

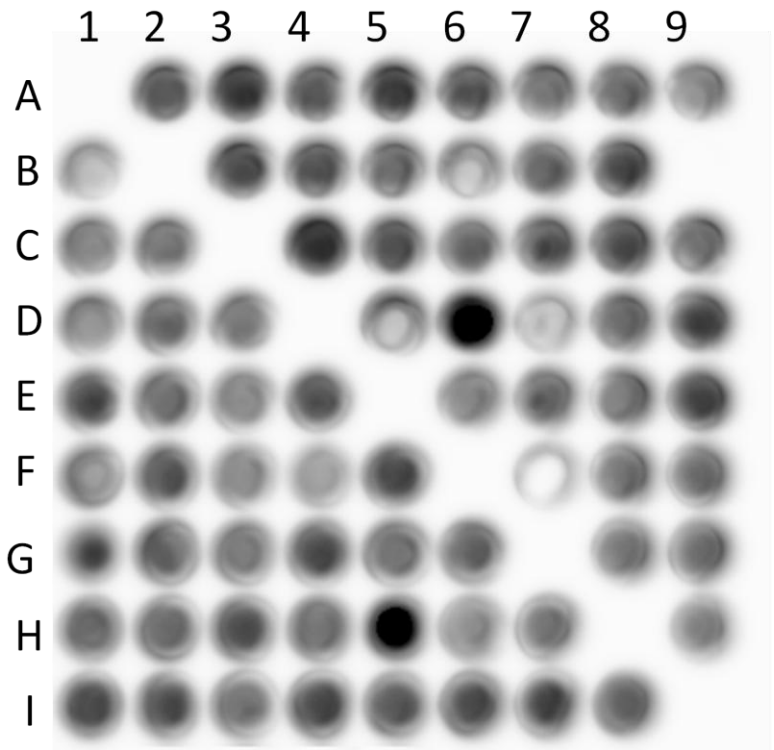


Figure 83 Chemiluminescent image of ELONA of MTase binding aptamers. Chemiluminescent image of 9 x 9 grid of MTase ELONA using candidate aptamers as set out in Figure 82. 5 µL 5 ug/mL MyOne beads were incubated with 1 µM of the designated biotinylated primary aptamer for 2 hours at 4°C, incubated with 1 µM MTase for 2 hours at 4°C, then 1 µM of the assigned secondary aptamer for 2 hours. The beads were incubated HRP streptavidin conjugate. ELONAs were developed and imaged using the Fujifilm Intelligent Dark Box reader.

The ELONA was undertaken with streptavidin coated paramagnetic beads as the immobilisation surface due to the inherent adaptability and ease of use. A grid was then constructed that contained combinations of two of each of the candidate aptamers that that have been selected against MTase (Figure 82). The relative intensities of each point on the grid was analysed using ImageJ software and the intensities plotted. The aim of the grid was to determine which of the pairs of aptamers could be used in conjunction with each other to create the best possible combination for a ELONA sandwich.

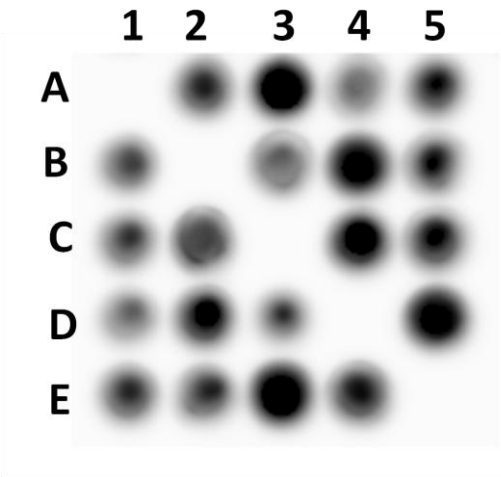


Figure 84 Figure 85 ELONA constructed from HsdR binding aptamers. Chemiluminescent image of 9 x 9 grid of HsdR ELONA using candidate aptamers as set out in Figure 82. 5 µL 5 ug/mL MyOne beads were incubated with 1 µM of the designated biotinylated primary aptamer for 2 hours at 4°C, incubated with 1 µM MTase for 2 hours at 4°C, then 1 µM of the assigned secondary aptamer for 2 hours. The beads were incubated HRP streptavidin conjugate. ELONAs were developed and imaged using the Fujifilm Intelligent Dark Box reader

Figure 83 and Figure 84 display that aptamers can be used in conjunction with each other in an ELONA sandwich. Although the intensity does vary between duplicate cells this can be attributed to uneven dispensing of magnetic beads, also athe controls highlight that the microplate used for this experiment contributed to non-specific signal of the HRP substrate. Time constraints prevented the completion of this area of the study, but it is a noteworthy addition to the biosensor concept.

6.4 Conclusions

In this chapter it has been demonstrated that the controlled release of a marker or further probe such as eGFP can be achieved using the modified ELISA system as a scaffold. This method will provide a suitable pre-cursor to the downstream applications of the biosensor, for example, if the eGFP is modified to the proposed alternative HsdR, (the molecular motor) the signal can be amplified sufficiently for a signal to be observed. The HsdR carries the same affinity attachment group, GST-transferase, and an identical proteolytic cleavage site, which would allow the substitution of the motor for eGFP. HsdR would then form a complex with HsdM in the configuration H_1M_2, and in the presence of ATP would recognise, bind to, and, translocate (Janscák *et al.*, 1998) the immobilised DNA substrate to produce a signal that is observable using the magnetic tweezers as described in the literature (Sisakova

et al., 2008). The potential for this as a biosensor is that it is a direct link between the biological and silicone worlds, as the proposed biosensor would produce a signal that carries very little background. This, coupled with the amplification method described here also allows the detection of analytes that is potentially far below current methodologies.

After multiple attempts using the modified ELONA method, it became clear that the streptavidin-Horse Radish Peroxidase conjugate which was acting as the probe for biotin was non-specifically binding the polystyrene assay plate. A limitation to the method became apparent in that the use of the paramagnetic beads provided a chance for the immobilisation surface to reduce. Further washes to reduce the non-specific binding also reduced the final signal indicating that additional washes also remove a significant proportion of the beads. Time constraints prevented the full exploration of the approach, however, initial runs of the procedure provide an insight into the benefits of this method.

7 Discussion

In this study it has been demonstrated that the isolation of aptamers through the SELEX process can be achieved using equipment and methodologies available to many molecular biology laboratories. It has also been shown that the analysis of aptamers can be achieved in a reliable repeatable and high throughput manner.

Though the analysis of the thrombin binding aptamer utilised techniques that may not be widely available, such as SPR , further work such as that performed on the HsdR binding aptamers have shown that this technique is not necessarily universally applicable as has been claimed. Affinity SELEX and GELEX have demonstrated the application of existing technologies to a novel problem, and as a consequence both methodologies have demonstrated the ability to enrich random aptamer libraries.

HPLC selection of Nitrotyrosine has demonstrated the enrichment of an aptamer library through progressive rounds of selection and whilst the study lacks a suitable assay to evaluate the efficacy of the enrichment process there was enough data to suggest successful enrichment in the form of novel sequences.

The dot blot method has proven to be the most applicable and versatile technology for the analysis of differing proteins, although this also limits the user to targets which bind to nitrotyrosine, or another similar membrane. The technique can probe many sequences in multiplex, and the high throughput manner of the method can even be used to give insight into binding affinities and kinetics.

Though the final experiment is missing from the series, where the aptamers are substituted for antibodies, evidence throughout is presented that enforces the notion that the substitution can be successful. Within the molecular amplifier, the protease cascade could release the molecular motor HsdR which would act as a nano-actuator translocating DNA in the presence of ATP and causing a positive signal on the optical tweezers for example.

A potential hurdle for the substitution of aptamers into the ELISA sandwich is the immobilisation of the aptamers onto a surface. Whether this is done through a biotin streptavidin linkage (which would be preferable), or through another attachment chemistry such as a terminal thiol linker to the functionalised surface could present some problems.

The surface may prevent the aptamers binding through steric interference or due to the target protein having some inherent charge that prevents the interaction from forming. This can be avoided through the application of polymeric linkers that move the aptamers away from the surface, which would serve well to avoid the problem of steric hindrance but the linker, may be detrimental to the binding of the aptamer to the target. The isolation of multiple sequences for each of the targets highlights the benefit of refinement of an aptamer library rather than a single binding motif. If a candidate aptamer is prevented from binding due to the conditions in which it was raised differing from the end point application, SELEX can be undertaken with the desired buffer conditions present throughout the selection process. This will result in an aptamer library that has been tailor made for the application for which it was intended.

An advantage of the HsdR aptamers is that the aptamers may bind to multiple epitopes of the protein. If an aptamer is found to inhibit one of the functionalities of the protein, another can be selected from the list that fulfils the criteria. This obstacle could potentially derail the substitution of aptamers for antibodies in the generic biosensor. The obstacle could be surmounted through the successful application of the ELONA assay, which allows the user to evaluate and avoid possible occupation of binding sites for other cofactors such as ATP. This application signifies one of the great advantages aptamers have over antibodies. The modular aspect of the list of potential aptamers allows the interchanging of a series of aptamers in the search for a suitable candidate that fits the specifications for the task. This, when coupled with the ability to produce large amounts of the oligonucleotide synthetically and bypassing the labourious task of producing antibodies *in vivo*, strengthens the case for aptameric molecular recognition elements. Aptamers also present another advantage over antibodies in the form of reproducibility. Due to the nature of the production of aptamers and the formation of distinct structural motifs, it can be assumed that aptamers of a known sequence will form a discreet number of structures of transition through them in a predictable way. As antibodies are the result of a long process drawn from animal immune responses that can take weeks to occur, the batch to batch efficacy of the antibody products hampers their use *in vitro* as each consignment needs to be assayed for affinity with known standards whenever binding needs to be examined.

Future work would involve the refinement of aptamer libraries to further proteins of interest, with a greater emphasis on the inhibitive and catalytic effects as part of the selection process. The use of aptazymes as a tailored molecular toolbox is restricted by the existence of a sensitive enough assay to enable to enrichment to select sequences that promote the desired effect. If a highly sensitive assay for the reaction already exists the random library can be subdivided to a size where such an effect would be observable. Another future direction would be the structural analysis of the complex formed during aptamer:antigen interactions. Through the use of Nuclear Magnetic Resonance, and protein crystallography data on the nature of the motif which the aptamers are folding to could give some insight into predesigned libraries that have some motifs surrounded by random regions, directing the library towards sequences that are similar to those that are known to bind. This may reduce specificity but, this may allow the user to enhance the power of *in vitro* evolution by providing a selection pressure towards an acknowledged goal.

In conclusion this work displays that aptamers are a useful tool that can work in conjunction and in place of antibodies as molecular recognition elements within biosensors, and that aptamers can be produced efficiently and reliably for many applications in most molecular biology laboratories.

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9 Appendices

9.1 Appendix 1

Examples of targets used for aptamer selections

Examples of targets used for aptamer selections	Target for aptamer selection	K_D	References
Inorganic components			
Zn2+	RNA	1.2 mmol/L	Ciesiolka et al. (1995)
Ni2+	RNA	0.8–29 mmol/L	Hofmann et al. (1997)
Small organic molecules			
Ethanolamine	DNA	6–19 nmol/L	Mann et al. (2005)
Theophylline	RNA	100 nmol/L	Jenison et al. (1994)
Malachite green	RNA	1 mmol/L	Grate and Wilson (2001)
Organic dyes	RNA DNA	100–600 mmol/L 33–46 mmol/L	Ellington and Szostak (1990) Ellington and Szostak (1992)
Sulforhodamine B	DNA	190 nmol/L	Wilson and Szostak (1998)
Hematoporphyrin	DNA	1.6 mmol/L	Okazawa et al. (2000)
Ricin toxin	DNA	58–105 nmol/L	Tang et al. (2006)
Cholic acid	DNA	5–67.5 mmol/L	Kato et al. (2000)

4,4'-Methylenedianiline	RNA	0.45–15 mmol/L	Brockstedt et al. (2004)
Dopamine	RNA	2.8 mmol/L	Mannironi et al. (1997)
Cocaine	DNA	n.s.[a]	Stojanovic et al. (2000)
Nucleotides and derivatives			
Adenine	RNA	10 mmol/L	Meli et al. (2002)
ATP (adenosine)	RNA	0.7–50 mmol/L	Sassanfar and Szostak (1993)
Adenosine/ATP	DNA	6 mmol/L	Huizenga and Szostak (1995)
ATP	RNA	4.8–11 mmol/L	Sazani et al. (2004)
Xanthine	RNA	3.3 mmol/L	Kiga et al. (1998)
cAMP	RNA	10 mmol/L	Koizumi and Breaker (2000)
Cofactors			
Coenzyme A	RNA	n.s.[a]	Saran et al. (2003)
Cyanocobalamin	RNA	88 nmol/L	Lorsch and Szostak (1994)
Riboflavin	RNA	1–5 mmol/L	Lauhon and Szostak (1995)
FMN	RNA	0.5 mmol/L	Burgstaller and Famulok (1994)
FAD	RNA	137–273 mmol/L	Burgstaller and Famulok (1994)

NAD	RNA	n.s.[a]	Burgstaller and Famulok (1994)
	RNA	2.5 mmol/L	Lauhon and Szostak (1995)
S-adenosyl methionine	RNA	n.s.[a]	Burke and Gold (1997)
S-adenosyl homocysteine	RNA	0.1 mmol/L	Gebhardt et al. (2000)
Biotin	RNA	5 mmol/L	Wilson et al. (1998) and Wilson and Szostak (1995)
Nucleic acids			
TAR RNA element of HIV-1	DNA	50 nmol/L	Boiziau et al. (1999)
	RNA	20–50 nmol/L	Duconge and Toulme (1999)
	DNA	50 nmol/L	Sekkal et al. (2002)
Yeast phenylalanine tRNA	RNA	12–26 nmol/L	Scarabino et al. (1999)
E.coli 5S RNA	RNA	6–12 mmol/L	Ko et al. (1999)
	RNA	3 mmol/L	Ko et al. (2001)
Amino acids			
L-Arginine	RNA	330 nmol/L	Geiger et al. (1996)
	DNA	2.5 mmol/L	Harada and Frankel (1995)
L-Citrulline	RNA	62–68 mmol/L	Famulok (1994)
L-Valine	RNA	12 mmol/L	Majerfeld and Yarus (1994)
L-Isoleucine	RNA	1–7 mmol/L	Lozupone et al. (2003)
	RNA	200–500 mmol/L	Majerfeld and Yarus (1998)
D-Tryptophan	RNA	18 mmol/L	Famulok and Szostak

			(1992)
L-tyrosinamide	DNA	45 mmol/L	Vianini et al. (2001)
L-histidine	RNA	8–54 mmol/L	Majerfeld et al. (2005)
Carbohydrates			
Cellobiose	DNA	600–nmol/L	Yang et al. (1998)
Sialyl Lewis X	RNA	0.085–10 nmol/L	Jeong et al. (2001)
Chitin	DNA	n.s.[a]	Fukusaki et al. (2000)
Sialyllactose	DNA	4.9 mmol/L	Masud et al. (2004)
Sephadex	DNA	n.s.[a]	Srisawat et al. (2001)
Antibiotics			
Kanamycin A	RNA	300 nmol/L	Lato et al. (1995)
Kanamycin B	RNA	180 nmol/L	Kwon et al. (2001)
Streptomycin	RNA	n.s.[a]	Wallace and Schroeder (1998)
Neomycin	RNA	100 nmol/L	Wallis et al. (1995)
Tobramycin	RNA	2–3 nmol/L	Wang and Rando (1995)
Lividomycin	RNA	300 nmol/L	Lato et al. (1995) and Lato and Ellington (1996)
Moenomycin A	RNA	300–400 nmol/L	Schu¨rer et al. (2001)
Tetracycline	RNA	1 mmol/L	Berens et al. (2001)
Chloramphenicol	RNA	25–65 mmol/L	Burke et al. (1997)
Peptides and proteins			
T4 DNA polymerase	RNA	5–30 nmol/L	Tuerk and Gold (1990)
α-Thrombin	DNA	200 nmol/L	Bock et al. (1992)
	RNA	<1–4 nmol/L	White et al. (2001)

Bovine thrombin	RNA	164–240 nmol/L	Liu et al. (2003)
Neurotensin receptor NTS-1 (rat)	RNA	0.37 nmol/L	Daniels et al. (2002)
Immunglobulin E	DNA	23–39 nmol/L	Mendonsa and Bowser (2004)
	RNA	30–35 nmol/L	Wiegand et al. (1996)
	DNA	10 nmol/L	Wiegand et al. (1996)
Interferon-g	RNA	2.7 nmol/L	Kubik et al. (1997)
MCP-1 (mouse)	RNA	180–370 pmol/L	Rhodes et al. (2001)
PDGF	DNA	0.1 nmol/L	Green et al. (1996)
VEGF	RNA	0.1–2 nmol/L	Jellinek et al. (1994)
	RNA	0.05–0.13 nmol/L	Ruckman et al. (1998)
HIV-1 integrase	RNA	10–800 nmol/L	Allen et al. (1995)
HIV-1 RT	RNA	5 nmol/L	Tuerk et al. (1992)
	DNA	1 nmol/	L Schneider et al. (1995)
	DNA	180–500 pmol/L	Mosing et al. (2005)
HIV-1 nucleocapsid protein	RNA	0.84–1.4 nmol/L	Kim et al. (2002)
TTF1	DNA	3.3–67 nmol/L	Murphy et al. (2003)
HGF	DNA	19–25 nmol/L	Saito and Tomida (2005)
Streptavidin	RNA	7 nmol/L	Tahiri-Alaoui et al. (2002)
	RNA	70–200 nmol/L	Srisawat and Engelke (2001)
	DNA	57–85 nmol/L	Stoltenburg et al. (2005)
L-Selectin	DNA	1.8–5.5 nmol/L	Hicke et al. (1996)
Taq DNA polymerase	DNA	0.04–9 nmol/L	Dang and Jayasena (1996)
Prion protein (PrPc)	RNA	0.1–1.7 nmol/L	Proske et al. (2002)
PrPSc fibrils	RNA	23.4 nmol/L	Rhie et al. (2003)
rPrPc	RNA	n.s.[a]	Weiss et al. (1997)
rPrPc	DNA	n.s.[a]	Takemura et al. (2006)

C5 protein	RNA	2–5 nmol/L	Biesecker et al. (1999)
Hepatitis C virus RdRp	DNA	1.3/23.5 nmol/L	Jones et al. (2006)
Hepatitis C virus NS3	RNA	n.s.[a]	Kumar et al. (1997)
Hepatitis C virus NS3 helicase	DNA	140 nmol/L	Zhan et al. (2005)
ppERK2/ERK2	RNA	4.7/50 nmol/L	Seiwert et al. (2000)
Protein kinase C delta	DNA	122 nmol/L	Mallikaratchy et al. (2006)
RNase H1	DNA	10–80 nmol/	L Pileur et al. (2003)
Colicin E3	RNA	2–14 nmol/L	Hirao et al. (2004)
Oncostatin M	RNA	7 nmol/L	Rhodes et al. (2000)
Tumour marker MUC1	DNA	0.1–34 nmol/L	Ferreira et al. (2006)
GnRH	L-RNA	190 nmol/L	Leva et al. (2002)
	L-DNA	45 nmol/L	
Vasopressin	L-DNA	1.2 mmol/L	Williams et al. (1997)
Amyloid peptide bA4(1-40)	RNA	29–48 nmol/L	Ylera et al. (2002)
Microcystin	DNA	1 mmol/L	Nakamura et al. (2001)
Complex structures			
Anthrax spores	DNA	n.s.[a]	Bruno and Kiel (1999)
Ribosomes/ribosomal protein S1	RNA	4-5 nmol/L	Ringquist et al. (1995)
Rous sarcoma virus (RSV)	RNA	n.s.[a]	Pan et al. (1995)
Differentiated PC12 cells	DNA	n.s.[a]	Wang et al. (2003)
Transformed YPEN-1 endothelial	DNA	n.s.[a]	Blank et al. (2001)

cells/pigpen			
U251 glioblastoma cells/tenascin-C	DNA	150 nmol/L	Daniels et al. (2003)
	RNA	5 nmol/L	Hicke et al. (2001)
Live African trypanosomes)	RNA	60 nmol/L	Homann and Go¨ringer (1999)
Jurkat T cell leukaemia	RNA	n.s.[a]	Lee and Lee (2006)
Receptors of host-cell matrix molecules on Trypanosoma cruzi	RNA	40–400 nmol/L	Ulrich et al., (2002)
Leukemia cells CCRF-	CEM DNA	0.8–229 nmol/L	Shangguan et al. (2006)

[a] n.s., not specified. (Stoltenburg et al., 2007)

9.2 Appendix 2

Designation	Description	References
Negative SELEX	- Minimizes the co-selection of unwanted nucleic acid ligands (e.g. for immobilization matrix) - Pre-selection with molecules, which should not be recognized, removing of unwanted oligonucleotide structures from the pool	Blank et al. (2001), Haller and Sarnow (1997), Vater et al. (2003) and Geiger et al. (1996)
Counter-SELEX or subtractive SELEX	- Generates aptamers which are able to discriminate between closely related structures - Introducing of a selection step	Jenison et al. (1994), Geiger et al. (1996) Haller and Sarnow (1997), Lee and Lee (2006), Shangguan et al. (2006), Wang

	to the related target for elimination of aptamers from the oligonucleotide pool, which are not able to distinguish between the related structures	et al. (2003) and White et al. (2003)
Blended SELEX -	- To give additional properties to aptamers beyond the binding capability - Enlarging of nucleic acid molecules by special non-nucleic acid components	Smith et al. (1995) and Radrizzani et al. (1999)
Expressions cassette SELEX or SELEX-SAGE	- Special forms of Blended SELEX (with transcription factors) - Optimizes aptamer activity for gene therapy applications	Martell et al. (2002) and Roulet et al. (2002)
Chimeric SELEX	Using of two or more different libraries for production of chimerical aptamers with more than one wanted feature or function - Each of the parent libraries will be selected first to a distinct feature, then fusion of the selected aptamers	Burke and Willis (1998)
Multi-stage-SELEX	- Special form of Chimeric SELEX - After fusion of preselected aptamer components, reselection to the entirety of	Wu and Curran (1999)

	the targets	
Deconvolution-SELEX	- To generate aptamers for complex - Discrimination between relevant aptamers (binding to distinct target structures within the complex mixture) and irrelevant oligonucleotides	targets Blank et al. (2001) and Morris et al. (1998)
Covalent SELEX or cross-linking SELEX	- Aptamers containing reactive groups which are capable of covalent linking to a target protein	Jensen et al. (1995) and Kopylov and Spiridonova (2000)
Photo-SELEX	- Aptamers bearing photo-reactive groups - Bind and photo cross-link to a target and/or photo activate a target molecule	Brody et al. (1999), Golden et al. (2000), Jensen et al. (1995) and Johnson and Gershon (1999)
SPIEGELMER®-Technology	- Selection with ordinary D-nucleic acids for the mirrored (enantiomer) target - Synthesis of the resulting aptamers as L-isomers which bind now to the originally un-mirrored target	Eulberg and Klussmann (2003), Klussmann et al. (1996) and Faulhammer et al. (2004)
Tailored-SELEX	- Integrated method to identify aptamers with only 10 fixed nucleotides through ligation and removal of primer binding	Vater et al. (2003)

	sites within the SELEX process - Useful for selection of short aptamers and Spiegelmers	
Signalling aptamers or molecular beacons	- Aptamers which report target binding by switching their structures and show a signal (e.g. fluorescent)	Jhaveri et al. (2000) and Rajendran and Ellington (2003)
Genomic SELEX or cDNA-SELEX	- Construction of a SELEX library of an organism's genome (e.g. cDNA fragments) - Target proteins or metabolites from the same organism are used to identify meaningful interactions - Allows the identification of protein targets directly from mRNA pools	Dobbelstein and Shenk (1995), Singer et al. (1997), Gold et al. (1997a,b), Zolotukhin et al. (2001), Chen et al. (2003), Shimada et al. (2005) and Wen and Gray (2004)
Toggle-SELEX	- Switching (''toggling'') between targets during alternating rounds of selection	White et al. (2001) andBianchini et al. (2001)
Indirect selection	- Selection target (e.g. metal ions) is not the actual binding partner of the aptamer, but binding is target-dependent (occurs only in presence of the target)	Kawakami et al. (2000)
In vivo selection	- One possibility to select RNA-processing signals - Uses transient transfection in	Coulter et al. (1997)

	an iterative procedure in cultured vertebrate cells	
Tissue SELEX	- Method for generating aptamers capable of binding to complex tissue targets such as collections of cells in diseased tissues	Daniels et al. (2003) and Morris et al. (1998)
TECS-SELEX	- Cell-surface displayed recombinant or natural protein is directly used as the selection target	Ohuchi et al. (2006) and Shangguan et al. (2006)
FluMag-SELEX	- DNA oligonucleotides with a fluorescein modification - Target immobilization on magnetic beads	Stoltenburg et al. (2005)
CE-SELEX	- Use of capillary electrophoresis (CE) For separation	Mendonsa and Bowser (2004), Mosing et al. (2005), Tang et al. (2006) and Drabovich et al. (2006)
Non-SELEX	- A process that involves repetitive steps of partitioning with no amplification between them - Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) used for partitioning	Berezovski et al. (2006)

EMSA-SELEX	- Use of electrophoretic mobility shift assay (EMSA) for partitioning in every round	Tsai and Reed (1998)
Use of nanoManipulatoratomic Force Microscope (nM-AFM)	- Only one round of selection necessary - Picking up and visualizing of singled aptamer target complexes by nM-AFM - Affinity measurement of single aptamer-target complexes possible by dynamic force spectroscopy - Amplification by single-molecule-PCR	Guthold et al. (2002)
On-chip selection	- Selection (in combination with a method for point mutations) and analyzing of DNA aptamers on chips	Asai et al. (2004)
Yeast genetic selection	- In vivo optimization of in vitro-preselected aptamers - Library with degenerated aptamers - Use of a yeast three(one)-hybrid system	Cassiday and Maher (2003)

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